Protective Action of Maternal Milk in Gastrointestinal Physiology

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Summary

Maternal milk is produced to nourish the newborn child and provide all nutrients required for ideal growth and development. Apart from delivering energy via the macronutrients lactose, fat and protein, milk contains multiple bioactive components that play a role in the immune development and response of the infant. These immunoregulatory factors are involved in diverse health benefits associated with breast-feeding. Among these beneficial human milk components are polyunsaturated fatty acids and human milk oligosaccharides. Especially the omega-3 and omega-6 long-chain polyunsaturated fatty acids fulfill important functions in brain and eye development and provide precursors for the eicosanoid lipid mediators controlling the immune system. Human milk oligosaccharides influence the immune system by acting as soluble receptors and binding to host proteins. Additionally, they shape the microbiota of the infant's gut by acting as prebiotics. This indirectly influences the action of the mucosal immune system. How long-chain fatty acids and milk oligosaccharides influence the intestinal microbiota and the immune response is the focus of this work.

Fatty acid composition of human milk and its change over the first month of lactation was determined by gas chromatography-mass spectrometry. While the relative content of most of the measured fatty acids is stable over the observed time period, it decreases for the omega-3 docosahexaenoic acid and steadily increases for its precursor α-linolenic acid. The higher docosahexaenoic acid levels right after birth might be crucial for the early brain development of the newborn and enhance the biosynthesis of anti-inflammatory lipid mediators. High levels of selected bioactive lipid mediators in human milk were newly discovered by liquid chromatography-tandem mass spectrometry. These data indicate that human milk delivers physiologically significant amounts of pro-inflammatory leukotriene B4 and anti-inflammatory and pro-resolving lipoxin A4, resolvin D1 and resolvin E1 and their hydroxy fatty acid precursors. Lipid mediator levels are stable with the exception of the direct precursor of resolvin D1, whose concentration declines over the first month postpartum. The high concentrations of anti-inflammatory and pro-resolving lipid mediators might lower the inflammatory state of the breast-fed infant.

The lactational change of selected human milk oligosaccharides over the first month postpartum was investigated by anion exchange chromatography with pulsed amperometric detection. For most milk oligosaccharides, the relative amount decreases over this time period. The most abundant milk oligosaccharide fucosyllactose shows stable levels over time, but high inter-individual differences. To study associations between neonatal fecal microbiota and the milk oligosaccharide levels of their mother's breast-milk, the fecal microbiota composition of corresponding breast-fed infants was

examined by denaturing gradient gel electrophoresis. Especially fucosyllactose might have a prebiotic effect, because high levels in human milk occurred together with the presence of a *Bifidobacteria* species in the infant feces. Fucosyllactose was further tested for its effects on microbial colonization and intestinal inflammation in a mouse supplementation study. Administration of fucosyllactose increased the intestinal content of *Barnesiella* in newborn mice. The ability of *Barnesiella* to utilize fucosyllactose as energy source was demonstrated in vitro. Additionally, fucosyllactose supplementation lowered the inflammatory response to dextran sulfate sodium-induced colitis in mice.

These results underline the uniqueness and bioactive potential of maternal milk. The tailor-made adjustment of docosahexaenoic acid concentration, the supply of anti-inflammatory lipid mediators and the potential of fucosyllactose to shape the microbiota and to lower the intestinal inflammatory response are beneficial features of human milk. They add to the health-promoting effects of human milk and cannot be mimicked by infant formula.

Zusammenfassung

Muttermilch wird erzeugt, um das neugeborene Kind zu versorgen und alle Nährstoffe bereitzustellen, die für ein optimales Wachstum und eine ideale Entwicklung benötigt werden. Neben ihrer Funktion als Energielieferant durch die Makronährstoffe Laktose, Fett und Protein, enthält Milch vielerlei bioaktive Komponenten, die eine Rolle bei der Entwicklung des Immunsystems und der Immunantwort des Säuglings spielen. Diese immunregulatorischen Faktoren sind Ursache verschiedener gesundheitlicher Vorteile, die mit Muttermilch in Verbindung gebracht werden. Zu diesen positiv wirkenden Muttermilchkomponenten gehören mehrfach ungesättigte Fettsäuren und Milcholigosaccharide. Besonders die langkettigen mehrfach ungesättigten omega-3- und omega-6-Fettsäuren erfüllen wichtige Funktionen in der Gehirn- und Augenentwicklung und liefern Vorstufen für die Biosynthese von Eicosanoid-Lipidmediatoren, die das Immunsystem kontrollieren. Humane Milcholigosaccharide beeinflussen das Immunsystem, indem sie sowohl als lösliche Rezeptoren fungieren als auch an körpereigene Proteine binden. Darüberhinaus formen sie die Mikrobiota des kindlichen Darms durch ihre Wirkung als Präbiotika. Dieses beeinflusst wiederum indirekt die Funktionsweise des mukosalen Immunsystems. In welcher Weise langkettige Fettsäuren und humane Milcholigosaccharide die intestinale Mikrobiota und die Immunantwort beeinflussen liegt im Fokus dieser Arbeit.

Die Fettsäurenkomposition der Muttermilch und ihre Veränderung innerhalb des ersten Monats der Laktation wurden mittels Gaschromatographie-Massenspektrometrie bestimmt. Während die meisten gemessenen Fettsäuren stabile Konzentrationen über den Beobachtungszeitraum aufweisen, sinkt der relative Gehalt der omega-3 Docosahexaensäure und steigt stetig für deren Vorstufe α-Linolensäure. Die höheren Gehalte an Docosahexaensäure direkt nach der Geburt könnten für die frühe neonatale Entwicklung des Gehirns entscheidend sein und die Biosynthese von anti-inflammatorischen Lipidmediatoren verstärken. Hohe Konzentrationen ausgewählter bioaktiver Lipidmediatoren in der Muttermilch wurden neu entdeckt mittels Flüssigchromatographie-Tandemmassenspektrometrie. Die Daten zeigen, dass Muttermilch physiologisch bedeutende Mengen des pro-inflammatorischen Leukotrienes B4 und der anti-inflammatorischen und entzündungsauflösenden Lipidmediatoren Lipoxin A4, Resolvin D1 und Resolvin E1 und deren Hydroxyfettsäuren-Vorstufen liefert. Die Level der Lipidmediatoren sind stabil mit der Ausnahme der direkten Vorstufe des Resolvin D1, dessen Konzentration während des ersten Monats nach der Geburt sinkt. Die hohen Konzentrationen anti-inflammatorischer und entzündungsauflösender Lipidmediatoren könnten den Entzündungsstatus des gestillten Säuglings herabsetzen.

Die Veränderungen ausgewählter Milcholigosaccharide während der Laktation wurden über den ersten Monat nach der Geburt mittels Anionenaustauscherchromatographie mit pulsierender amperometrischer Detektion untersucht. Der relative Gehalt der meisten Milcholigosaccharide sinkt über diesen Zeitraum. Der am reichlichsten vorhandene Milcholigosaccharid Fucosyllaktose weist zeitlich stabile Level auf, dafür jedoch grosse interindividuelle Unterschiede. Um den Zusammenhang zwischen fekaler Mikrobiota eines Neugeborenen und der Milcholigosaccharidkonzentrationen seiner Mutter zu untersuchen, wurde die Zusammensetzung der fekalen Mikrobiota der zugehörigen gestillten Säuglinge mittels denaturierender Gradientengelelektrophorese bestimmt. Besonders Fucosyllaktose könnte einen präbiotischen Effekt haben, da hohe Level in der Muttermilch gleichzeitig mit dem Vorkommen einer Bifidobakterienspezies im Feces des Kindes auftraten. Fucosyllaktose wurde daraufhin auf ihre Effekte auf die mikrobielle Darmkolonisation und intestinale Entzündung in einer Maussupplementationsstudie untersucht. Die Gabe von Fucosyllaktose erhöhte den intestinalen Anteil von *Barnesiella* bei neugeborenen Mäusen. Die Fähigkeit, Fucosyllaktose als Energiequelle zu nutzen, wurde für *Barnesiella* in vitro bestätigt. Die Supplementation von Fucosyllaktose führte bei den Mäusen zusätzlich zu einer verminderten Entzündungsantwort auf Natrium-Dextransulfat induzierte Colitis.

Diese Ergebnisse unterstreichen die Einzigartigkeit und das bioaktive Potential der Muttermilch. Die bedarfsgerechte Anpassung der Docosahexaensäurekonzentration, die Versorgung mit anti-inflammatorischen Lipidmediatoren und das Potential von Fucosyllaktose, die Mikrobiota zu formen und die intestinale Immunantwort zu vermindern, sind positiv wirkende Eigenschaften der Muttermilch. Diese tragen zu den gesundheitsfördernden Effekten der Muttermilch bei und können nicht von Säuglingsmilchnahrung nachgeahmt werden.

Abbreviations

12-HETE, 12-hydroxyeicosatetraenoic acid; **15-HETE**, 15-hydroxyeicosatetraenoic acid; **17-HDHA**, 17-hydroxydocosahexaenoic acid; **18-HEPE**, 18-hydroxyeicosapentaenoic acid; **2FL**, 2-fucosyllactose; **3FL**, 3-fucosyllactose; **3SL**, α2,3-sialyllactose; **6SL**, α2,6-sialyllactose; **AA**, arachidonic acid; **ACN**, acetonitrile; **DC**, dendritic cell; **DGGE**, denaturing gradient gel electrophoresis; **DHA**, docosahexaenoic acid; **DSS**, dextran sulfate sodium; **EPA**, eicosapentaenoic acid; **Gal**, galactose; **GalNAc**, N-acetylgalactosamine; **GC-MS**, gas chromatography mass spectrometry; **Glc**, glucose; **GlcNAc**, N-acetylglucosamine; **GU**, glucose units; **HIV**, human immunodeficiency virus; **HPAEC-PAD**, high-performance anion exchange chromatography-pulsed amperometric detection; **HPLC-MS/MS**, high-performance liquid chromatography tandem mass spectrometry; **IL**, interleukin; **KO**, knockout; **LCPUFA**, long-chain polyunsaturated fatty acid; **LTA4**, leukotriene A4; **LTB4**, leukotriene B4; **LXA4**, lipoxin A4; **MALDI-TOF-MS/MS**, matrix assisted laser desorption ionization-time of flight-tandem mass spectrometry; **NEC**, necrotizing enterocolitis; **NeuAc**, N-acetylneuraminic acid; **PGD2**, prostaglandin D2; **PGE2**, prostaglandin E2; **PGF2α**, prostaglandin F2α, **PGI2**, prostacyclin; **PUFA**, polyunsaturated fatty acid; **RvD1**, resolvin D1; **RvE1**, resolvin E1; **Sia**, sialic acid; **SPE**, solid phase extraction; **TFA**, trifluoroacetic acid; **TXA2**, thromboxane A2

Introduction

Human milk is the natural creation of a food that contains everything a newborn child needs. Therefore, breast-feeding is regarded as gold standard for the infant‘s nutrition. It is associated with various beneficial effects on the newborn’s health. In breast-fed children, the incidence of allergic and inflammatory conditions is decreased. The question that is intensely investigated but still not fully answered is which components of human milk influence the breast-fed child, so that it develops advantages in growth, health status and immunity over formula-fed children.

1 Breast milk versus formula – what are the differences?

1.1 Effects on health

Breast-fed children have evidently certain health advantages in comparison to formula-fed children (1). In several studies, beneficial effects of breastfeeding were shown on different levels. For many autoimmune diseases for example, the risk of developing the disease is reduced by breastfeeding. Type 1 diabetes occurs after autoimmune destruction of the pancreatic beta cells and its prevalence has steadily increased (2). The risk of developing this type of diabetes is lower in breast-fed children than in children fed infant formula (3). Furthermore, the development of coeliac disease, an autoimmune disorder triggered by gluten protein and resulting in inflammation and destruction of the intestinal lining, is negatively correlated with the duration of breastfeeding (4).

Also, breast-feeding influences the onset ot atopic diseases like atopic dermatitis and atopic asthma. Exclusive breast-feeding for three months lowered the susceptibility to atopic dermatitis (5, 6) and childhood asthma (7-9), especially in families with a history of atopy. The risk of food allergies is as well reduced with longer breastfeeding. Children who were breast-fed for less than one month or not at all had a significantly higher incidence of food allergy at the age of 1 to 3 years than children breast-fed for more than one month (10).

Moreover, breastfeeding plays a protective role in several medical disorders that occur generally in adulthood and in combination are known as the metabolic syndrome which is associated with increased risk for cardiovascular disease (11, 12). These disorders include obesity, hyperglycemia, dyslipidemia and hypertension. In several studies, a lower risk of obesity in adulthood has been confirmed for breast-fed children (13-15). Also type 2 diabetes, often accompanying obesity and characterized by insulin resistance, occurs less often in breast-fed children compared to formula-fed ones (16). One symptom of dyslipidemia is a low content of high density lipoprotein cholesterol

together with a higher content of low density lipoprotein cholesterol in the blood (17). Breastfeeding entails a reduced risk of dyslipidemia, since total cholesterol as well as low density lipoprotein cholesterol seem to be lower (18), while high density lipoprotein cholesterol was shown to be higher in adults that had been breastfed (12). In addition, the fourth component of the metabolic syndrome, hypertension, seems to be less likely in later life of the breastfed child according to several studies (19). The blood pressure, systolic more than diastolic, was shown to be significantly lower when breastfed after birth (19). But this effect is rather modest and seems to be negligible when considering future health outcomes (20).

A matter of controversial debate is the effect of infant feeding mode on the intelligence of the child. While a positive effect of breastfeeding on the intelligence quotient is found in some studies (21-23), it is not confirmed by others in which it was shown that the link between breastfeeding and cognitive abilities is rather due to confounding factors like maternal intelligence quotient (24).

The direct impact of breast milk is probably observed in the digestive tract of the newborn, the site of direct contact and interaction with the mucosal epithelium as well as with the intestinal microbiota. These firsthand effects of breast milk are less controversial than the observed long-term influences. For example, the beneficial consequences of breastfeeding in the development of the mucosal immune system are evident. When comparing different feeding modes, a major difference appears in the occurrence of gastrointestinal infections resulting in diarrhea. Morbidity and mortality due to diarrhea is reduced in breast-fed infants (25). In diarrhea caused by infections with *Campylobacter jejuni*, attack rates in the first six months of age were shown to be 2.3-fold higher in infants that were not fed breast milk (26). In a study from Bangladesh, reduced mortality resulting from diarrhea was confirmed for breast-fed infants (27). Here, the risk of death from diarrhea was almost 4-fold higher in partially or non-breast-fed infants. Considering that the study was conducted with infants born in slum areas, the lower risk associated with exclusive breast-feeding might partly be attributed to the lower exposure to contaminated food and drinking water. Apart from preventing infections, breast milk also reduces the risk of inflammatory bowel diseases like Crohn's disease (28). Also necrotizing enterocolitis (NEC), necrosis of bowel tissue that is primarily observed in preterm neonates, occurred less often in preterm infants who were fed human milk (29, 30). Feeding human milk is associated with a six-fold lower risk for NEC in low birth weight infants (31).

Feeding breast milk seems to entail many advantages for the growth and development of the neonate and the key to these functional benefits is a plethora of health-promoting compounds that are contained in human breast milk.

1.2 Composition of human milk and infant formula

Although the composition of infant formula is made to imitate breast milk, it still differs considerably from maternal milk. In Switzerland, the mandatory concentrations of the macronutrients carbohydrates, fat and protein of infant formula are concordant with the one of mature term breast milk (Table 1). But looking at the molecular structures inside these categories carbohydrates, fat and protein reveals the actual differences between human milk and formula.

Table 1: Macronutrient (g/dl) and energy (kcal/dl) composition of human milk (adapted from (32))

	Protein Mean (±2 SD)	Fat Mean (±2 SD)	Lactose Mean (±2 SD)	Energy Mean (±2 SD)	Ref.
Term infants, 24-h collection, mature milk (3 months postpartum)					
	1.2 (0.9, 1.5)	3.6 (2.2, 5.0)	7.4 (7.2, 7.7)	70 (57, 83)	(33)
Donor human milk samples					
United States milk bank donors	1.2 (0.7, 1.7)	3.2 (1.2, 5.2)	7.8 (6.0, 9.6)	65 (43, 87)	(34)
Danish milk bank donors	0.9 (0.6, 1.4)[a]	3.6 (1.8, 8.9)[a]	7.2 (6.4, 7.6)[a]	67 (50,115)[a]	(35)
Preterm (23 to 33 weeks' gestational age), 24-h collection, first 8 weeks of life					
Born <29 weeks	2.2 (1.3, 3.3)	4.4 (2.6, 6.2)	7.6 (6.4, 8.8)	78 (61, 94)	(36)
Born 32–33 weeks	1.9 (1.3, 2.5)	4.8 (2.8, 6.8)	7.5 (6.5, 8.5)	77 (64, 89)	(36)
Preterm donor milk					
	1.4 (0.8, 1.9)	4.2 (2.4, 5.9)	6.7 (5.5, 7.9)	70 (53, 87)	(37)
Swiss regulations for infant formula composition					
	1.1-2.1[b]	2.6-4.2[b]	>2.9[c]	60-70[b]	(38)

a Median (lower 2.5 percentile, upper 97.5 percentile).
b Minimum and maximum value.
c Total amount of carbohydrates 5.4-9.8 g/dl.
SD, standard deviation; Ref., Reference.

1.2.1 Proteins

The protein fraction of mature human milk consists of 60% whey protein and 40% casein (39). The whey protein of human milk comprises many bioactive proteins like immunoglobulins, lactoferrin and lysozyme that have antimicrobial activity. The high percentage of acid soluble whey protein and the low percentage of acid insoluble casein eases the digestion for the newborn (40). The protein in cow's milk has only 20% whey protein and 80% casein (41), which makes it more difficult to digest. In addition, the total protein content of cow's milk is 3.3% (42), hence, almost three times higher than in human milk. Therefore, former infant formulas based on cow's milk had a very dissimilar protein profile to human milk. But nowadays, the protein content and also the corresponding share of whey

and casein is controlled in formula production and is generally adjusted to the values of human milk (43).

1.2.2 Carbohydrates

The carbohydrate fraction in breast milk as well as in infant formula is mainly constituted of lactose. But in formula, there often is a considerable amount of starch added, to thicken the formula to reduce regurgitation, for example. But most importantly, formula miss a significant part of carbohydrates that are unique for human breast milk, namely the human milk oligosaccharides. These oligosaccharides are present in a vast structural variety and in concentrations of up to 23 g/l breast milk (44). The amount and also the number of different structures is lower in cow's milk (45, 46) and in milk from other mammals (46, 47). Therefore, many milk oligosaccharides unique for human milk are not available for supplementation of infant formula so far (48). Nowadays, a few types of formula are fortified with oligosaccharides that are structurally different to human milk oligosaccharides, though. But these galacto- and fructooligosaccharides seem to affect the microbiota in such a way that it is more similar to the microbiota of breast-fed infants (49, 50).

1.2.3 Fat

Fat is not only the main energy source of breast milk, it also fulfills many functions in the development and growth of the infant. Especially, the polyunsaturated fatty acids (PUFAs) are important for the nervous tissue, visual system development and eicosanoid synthesis (51). The fatty acid composition of formula used to deviate a lot from the one of human milk. The biggest difference used to be the content of long-chain PUFAs (LCPUFAs). Human milk contains up to 5% of the total fat content as omega-3 and omega-6 LCPUFAs (52). While LCPUFAs were not added to infant formula in earlier times, they usually are nowadays in amounts of circa 4% of the total fat content (53-55), although the addition is not mandatory according to the Swiss law (38). Especially, docosahexaenoic acid (DHA) and arachidonic acid (AA) got a lot of attention after it was proven to be present in breast milk in quite considerable amounts (56). In 2001, the US Food and Drug Administration approved the claim and considered DHA and AA as generally recognized as safe (57). From 2002, DHA and AA were added to some infant formula (58).

1.2.4 Immunomodulatory factors in human breast milk

In general, nutrition can influence the development of the immune system of young infants in several ways (59). On the one hand, it is a source of antigens that train the immune system to develop

tolerance. On the other hand, it delivers substances that modulate processes of the immune system either directly or indirectly. The indirect way comprises an effect on the intestinal microbiota that as well can modulate the immune response (60). For instance, alterations of the core microbiome of the body can lead to diseases like Crohn's disease, NEC, allergy and susceptibility to infections (61).

Human breast milk delivers plenty of substances that are potent immune-regulators to support the innate and also the not yet fully developed adaptive immunity of the newborn, in a direct way or indirectly via the gut microbiota (62). Components of the adaptive immune system that are transferred from the mother to the newborn are immunoglobulins. In human milk, more than 90% of the immunoglobulins are present as secretory immunoglobulin A (63). Secretory immunoglobulin A inhibits the adherence of enteric pathogens and thereby lowers infections (62). The innate immune system of human milk comprises many proteins with antimicrobial activity, including κ-casein, α-lactalbumin, lactoferrin, haptocorrin and lysozyme (63). κ-Casein is a highly glycosylated protein in human milk that binds to *Helicobacter pylori* and reduces its adhesion to the mucosal cell (64). After partial proteolysis in the gastrointestinal tract, the polypeptide fragments of α-lactalbumin present antimicrobial activity against several pathogens (65). Apart from diverse antibacterial and antiviral effects, Lactoferrin influences bacterial colonization by rendering iron unavailable for iron-requiring pathogens, and haptocorrin is able to inhibit the growth of enterotoxigenic *Escherichia coli* (62). The enzyme lysozyme in human milk protects against bacteria by breaking down peptidoglycans of the bacterial cell wall (66). This leads to cell lysis and destroys Gram-positive as well as Gram-negative bacteria (67).

While the human milk proteins described above protect the newborn by acting on pathogenic bacteria or viruses, other proteins support the newborn's immunocompetence, the ability to develop an immune response. This immune response can be mediated by the action of protein hormones such as cytokines. Human milk contains a number of cytokines including interleukin (IL)-1, IL-1β, IL-6, IL-8, IL-10, tumor necrosis factor-α, transforming growth factor-β and interferon-γ (68). They are present in their free form, but are also secreted by maternal immune cells that are contained in human milk (63). Also the corresponding soluble receptors have been detected in human milk (69). These cytokines have pro- or anti-inflammatory effects (32). The neonatal production of some cytokines is reduced (68). Thus, a supply through breast milk might ascertain a proper regulation of inflammatory and immune responses despite the developmental delay of the neonatal immune system.

The composition of these immune-regulating molecules is one of the major differences when comparing the nutrients of breast milk to the one of infant formula. There have been many immunomodulatory factors identified in human milk to date and the number is still expanding. Milk

oligosaccharides have been identified as one of them (62). The high levels of PUFAs in human milk might also play a role in the positive effect of human milk on the immune system. The influence of these two human milk components, oligosaccharides and fatty acids, especially polyunsaturated ones, on the structure of the intestinal microbiota and on the immune system are the focus of this work.

2 Lipids

2.1 Human milk fat

Human milk contains approximately 30-50 g fat per liter. However, the fat concentration of human milk is highly variable. It varies greatly between individuals and from early to late lactation (30, 70). Some studies also describe changes in the fat content over the day (71, 72). Feedings in the night and morning were found to deliver milk with a lower fat content than in the afternoons and evenings (71). During one feed, the initial part of the milk, so-called foremilk, contains two to three times less fat than hindmilk, the second part of the milk (70). The reason is the constant fat production in the mammary gland providing the same absolute amount of fat with simultaneously decreasing milk volume during breast emptying (73).

Human milk fat is present as fat droplets (74). These fat droplets consist of a core surrounded by a lipoprotein membrane. The core mainly comprises mono-, di- and triglycerides and free fatty acids. The fat globule membrane is built up of phospholipids, cholesterol and cholesteryl ester. It also contains a variety of glycolipids, gangliosides, glycoproteins and mucins. Looking at the total distribution of lipids in human milk, almost all (circa 97%) are triglycerides (75). Mono- and diglycerides account for circa 0.2% and 0.5%, respectively. Approximately 0.6% of the milk lipids are free fatty acids. The remaining percentages cover the membrane lipids with roughly equal concentrations of phospholipids and cholesterol. Cholesteryl esters account for 20% of cholesterol.

Human milk fat is the main energy source for the breast-fed newborn. Fifty to sixty percent of the calories in human milk derive from fat (74). Since 97% of the milk fat are triglycerides of which 90% are fatty acids, 88% of human milk fat are esterified fatty acids (74). Besides delivering energy, the different fatty acids fulfill several functions.

The most abundant fatty acids in human milk fat are oleic acid (C18:1) and palmitic acid (C16:0), followed by linoleic acid (C18:2) (75, 76). These three fatty acids account for 70-80% of the total fatty acid content. The remaining 20-30% are mainly represented by stearic acid (C18:0), palmitoleic acid

(C16:1), AA (C20:4), α-linolenic acid (αC18:3), and DHA (C22:6). For the function of fatty acids, the degree of saturation is important (77). In human milk, 43% of the fatty acids are saturated, 43% are monounsaturated and 14% are polyunsaturated (76).

Saturated fatty acids mainly serve as energy source. The long-chain saturated fatty acids C22:0 and C24:0 are, together with C18:0, components of the myelin sheath in the nervous system (77, 78). Monounsaturated fatty acids are important membrane components and ensure, for example, transporter translocation and insulin receptor affinity (79, 80). Immune effects of monounsaturated fatty acids shown in animals have not been confirmed in humans yet (81).

Breast milk is rich in PUFAs. PUFAs are crucial components of membrane phospholipids. They influence the membrane fluidity and act as ligands for membrane receptors and transcription factors, thereby enabling cellular communication and gene expression (83). The omega-6 and omega-3 PUFAs play central roles in immune and inflammatory pathways.

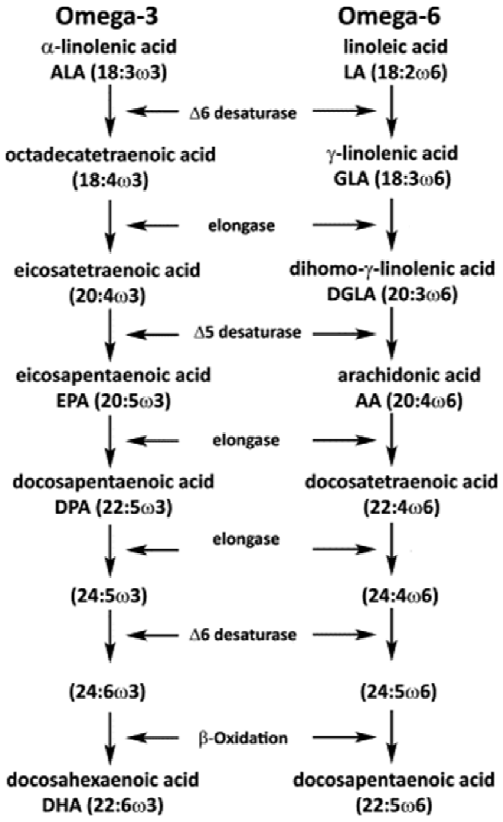

Figure 2.1: Synthesis of omega-3 and omega-6 PUFAs from linolenic and linoleic acid (adapted from (82)).

The major omega-6 and omega-3 fatty acids in human milk are linoleic (C18:2n-6) and α-linolenic acid (αC18:3n-3). They are the two essential fatty acids, i.e. they have to be provided by the diet, because they cannot be produced by the human fat metabolism. The human body is not able to create double bonds in the omega-6 and omega-3 positions of long-chain fatty acids, because it lacks the required Δ12 and Δ15 desaturase enzymes (83, 84). Thus, dietary linoleic and linolenic acid provide precursors for the human biosynthesis of all omega-6 and omega-3 fatty acids, respectively (Figure 2.1). Since they are not interconvertible by the human metabolism, both linoleic and linolenic acid are essential. Linoleic acid is the precursor of AA (C20:4n-6) and linolenic acid the precursor of eicosapentaenoic acid (EPA, C20:5n-3) and DHA (C22:6n-3). The supply of these three LCPUFAs is assured by biosynthesis and dietary intake. In adults, they can be consumed in animal tissues like fatty fish (83). For the newborn, they are supplied by human milk or special infant formulas in addition to their own production. However, the biosynthesis in newborns seems to be

less efficient than in adults (85). In that case, the nutritional supply should be guaranteed since AA, DHA and EPA are important for growth and development.

DHA and AA are needed for normal development of brain, retina and the nervous system (86, 87). DHA and AA are the major polyunsaturated fatty acids of neural tissues, and DHA is the most abundant fatty acid in the retina (88, 89). Due to lower DHA content in infant formula than in breast milk, formula-fed infants have a lower cerebral content of DHA, but not of AA, than breast-fed infants (90, 91). The omega-3 fatty acids EPA and DHA play protective roles in cardiovascular health. A higher EPA and DHA content in red blood cell membranes is associated with a decreased onset of coronary heart diseases (92). The DHA content of red blood cells is also lower in formula-fed infants than in breast-fed infants (84). Additionally, LCPUFAs are precursors of pro- and anti-inflammatory lipid mediators. These lipid mediators are hormone-like substances that play an important role in the process of inflammation. Breastfeeding reduces the occurrence of inflammation in the newborn, for example in the respiratory and gastrointestinal tract. This protective effect of breast milk could be mediated by delivering lipid mediator precursors, in form of LCPUFAs.

The anti-inflammatory potential of LCPUFAs was shown in the treatment of several inflammatory and autoimmune diseases (93), like inflammatory bowel diseases (94). Administration of EPA led to an improvement of ulcerative colitis (95, 96). In Crohn's disease, omega-3 LCPUFAs could reduce the rate of relapse (97). LCPUFA also reduces the risk of developing NEC. The supplementation of infant formula with LCPUFA resulted in a lower incidence of NEC in rats (98). Moreover, the lower incidence of childhood allergy and eczema in breast-fed infants compared to formula-fed ones may be attributed to the beneficial effects of the LCPUFAs in breast milk (99).

DHA and other LCPUFAs are accumulated primarily in the last ten weeks of pregnancy and the first year of life (100). In pregnancy, the transplacental transfer is in favor of DHA over other fatty acids during the last trimester (101). Intrauterine accretion in this time was shown to be 43 mg/kg/day of DHA and 212 mg/kg/day of AA (102). Preterm infants that lack this period of LCPUFA accumulation have higher dietary requirements after birth, because their synthesis at one month of age with 13 mg/kg/day DHA and 27 mg/kg/day AA do not meet the requirements defined by the fetal accretion rates (103). In view of this mismatch, AA and DHA is discussed to be conditionally essential, with one of these conditions being prematurity (104). Interestingly, the DHA content of preterm milk is higher than in term milk (105) which matches the elevated need of preterm infants for DHA.

Several factors influence the fatty acid composition of breast milk. Next to lactational and gestational age, diseases and individual factors have an influence (106). But the greatest effect comes from the maternal diet, which also accounts for seasonal and geographical differences. In a study in China, the

breast milk fatty acid composition of women from urban, rural, pastoral and marine regions were compared (107). The biggest difference was the concentration of DHA. While in urban, rural and pastoral areas, women had a DHA content between 0.1 and 0.9 wt%, it was 2.78 wt% in breast milk from marine regions. The comparison of the diet revealed a significantly higher intake of seafood and also soybean oil in the marine region. The DHA content in breast milk can also be increased by supplementation (108). The daily intake of DHA increased the DHA concentration in human milk in a dose-dependent manner. The content of omega-3 fatty acids could be increased by fish oil supplementation during lactation (109). Women who got fish oil containing a mix of DHA and EPA had three times higher concentrations of omega-3 fatty acids in their breast milk. The dependency of the milk fatty acid composition on the maternal diet was already demonstrated earlier when a high consumption of corn oil with a linoleic acid content of 52% led to an increased linoleic acid concentration in breast milk of up to 43% (110). The fact that increased maternal PUFA intake raises PUFA concentration in breast milk can be applied advantageously to guarantee the optimal supply to the newborn. For example, women with low DHA intake are recommended DHA supplementation for the time of breastfeeding (111, 112).

2.2 Lipid mediators

Human milk contains multiple bioactive components. Next to oligosaccharides, immunoglobulins, growth factors, immune cells and cytokines, also lipids are relevant for the immune system development. LCPUFAs have been shown to be immunoregulatory (113). The molecular basis of their beneficial actions might be the LCPUFA derivatives, the so-called lipid mediators. They are produced by the combined action of lipoxygenases, cyclooxygenases, hydrolases and cytochrome P450 (Figure 2.2 and Figure 2.3). In many inflammatory disorders of the gastrointestinal tract, neutrophil migration into the mucosa parallels epithelial injury. Lipid mediators significantly influence these leukocyte-epithelium interactions. They have hormone-like functions and are crucial for the process of inflammation. Their effects can be pro- or anti-inflammatory, i.e. they regulate the onset or the resolution of inflammation. Their presence in human milk is unknown so far.

2.2.1 The onset of inflammation – Pro-inflammatory lipid mediators

Acute inflammation is the response to tissue injury, infection with microorganisms or surgical trauma (114). Also restoration of blood flow after its complete or partial arrest as in ischemia-reperfusion injury can trigger inflammation. The process starts with the release of chemical mediators that act as chemoattractants and initiate the recruitment of neutrophils to the site of inflammation. As the first line of defence, the neutrophils infiltrate the inflamed tissue and are able to phagocytise cellular

debris and invading microorganisms. The chemoattractant mediators are either exogenous, for example antimicrobial peptides, or endogenous, for example eicosanoids. The endogenous chemical mediators that promote acute inflammation are released by neutrophils migrating into the tissue. They include, next to cytokines and chemokines, also lipid mediators like prostaglandins, thromboxanes and leukotrienes (115).

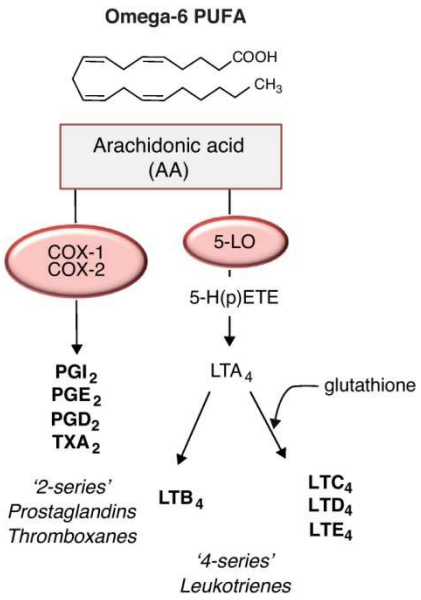

Figure 2.2: Arachidonic acid as precursor for pro-inflammatory prostaglandins, thromboxanes and leukotrienes (cited from (116)). COX, cyclooxygenase; PG, prostaglandin; PGI, prostacyclin; TX, thromboxane; 5-LO, 5-lipoxygenase; 5-H(p)ETE, 5-Hydro(pero)xyeicosatetraenoic acid; LT, leukotrienes

The 2-series prostanoids, including prostaglandins, prostacylins and thromboxanes, and the 4-series leukotrienes are derived from AA via cyclooxygenases and lipoxygenases, respectively (Figure 2.2). The same enzymes convert EPA to 3-series prostanoids and 5-series leukotrienes (116). However, these eicosanoids show a lower bioactivity and are less pro-inflammatory (117, 118).

Both isoforms of cyclooxygenase, COX-1 and COX-2, produce prostaglandin H_2 which serves as common precursor for the synthesis of thromboxane A_2 (TXA_2) and the four principal bioactive prostaglandins prostaglandin E_2 (PGE_2), prostaglandin D2 (PGD_2), prostacyclin (PGI_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (119). One of the most abundant prostaglandins is PGE_2. This mediator plays a dual role exerting pro- and anti-inflammatory responses. It regulates cytokine expression of dendritic cells and stimulates their migration to draining lymph nodes, and thus, enhances the activation of T cells (120, 121). In contrast, its influence on innate immune cells can be anti-inflammatory, for example the initiation of the anti-inflammatory lipid mediator biosynthesis in neutrophils (122).

PGD_2 plays a pro-inflammatory role in allergic inflammation, especially acute allergic responses as seen in asthma, and seems to contribute to the development of atherosclerosis (119). Contrarily, it inhibits dendritic cell migration and the proliferation of T cells (123), thereby reducing the severity of the inflammatory response. $PGF_{2\alpha}$ is a biomarker of oxidative stress and inflammation. Elevated levels of this mediator have been associated to many pathophysiological conditions (124). The mediators PGI_2 and TXA_2 have some opposing functions. PGI_2 inhibits platelet aggregation and causes vasodilatation (125), whereas TXA_2 promotes platelet adhesion and aggregation and acts as

vasoconstrictor (126). In addition, PGI_2 is described as anti-inflammatory and immunosuppressive, while TXA_2 rather shows pro-inflammatory activities.

Leukotrienes are generated by 5-lipoxygenase action on AA yielding leukotrienes A_4 (LTA_4) (Figure 2.2). The conjugation of LTA_4 with glutathione yields the cysteinyl leukotrienes LTC_4, LTD_4 and LTE_4. Together, they build the slow reacting substance of anaphylaxis that causes immediate hypersensitivity and bronchoconstriction (127). Apart from this function, they seem to play a role in chronic inflammatory cell responses (128). Leukotriene B_4 (LTB_4) is generated from LTA_4 by LTA_4 hydrolase (115) and plays a crucial role in acute inflammation. It is a potent chemoattractant that activates firm adhesion of leukocytes and initiates their migration (129). Additionally, it induces the production of reactive oxygen species in neutrophils and inhibits neutrophilic apoptosis (130, 131).

The inflammatory response is a well-orchestrated process of many cascades initiated by these specific pro-inflammatory lipid mediators that have distinct and partially overlapping effects. Some of the described pro-inflammatory lipid mediators are also able to restrain acute inflammation. Apart from them, some anti-inflammatory and pro-resolving lipid mediators are specialized on the task of bringing inflammation to an end (114).

2.2.2 The resolution of inflammation – Anti-inflammatory lipid mediators

Not only the initiation of inflammation, but also its resolution is an active process (132). The inflammatory response is the first line of defense and protects the host, but it needs to be self-limiting to prevent consequences of exaggerated inflammation. Uncontrolled, excessive neutrophil infiltration increases inadvertent spilling of neutrophil granule content (133). This granule content contains degenerative enzymes and reactive oxygen species that can cause tissue damage. Another undesired outcome is the development of chronic inflammation. Thus, the regeneration of homeostasis, i.e. the resolution of inflammation, needs to be regulated.

During the process of acute inflammation, the pro-inflammatory PGE_2 and PGD_2 also show pro-resolution activities. They change the expression of enzymes in neutrophils to generate anti-inflammatory lipid mediators. They stimulate the lipid mediator class switch from pro- to anti-inflammatory with the result that concentrations of anti-inflammatory and pro-resolving lipid mediators rise (114, 134). Those include lipoxins, resolvins, protectins/neuroprotectins and maresins. They oppose many actions of and also inhibit pro-inflammatory mediators with the aim to stop or prevent inflammation (114). Their biosynthesis is done either via the lipoxygenase metabolism (Figure 2.3), resulting in the S-series lipid mediators, or by an aspirin-triggered cyclooxygenase or cytochrome P450 metabolism (135, 136). From the aspirin-triggered pathway, a second series of lipid

mediators evolves: the R-lipoxins, R-resolvins, R-protectins and R-neuroprotectins. These aspirin-triggered lipid mediators have anti-inflammatory potential which is equivalent to the S-series mediators (136).

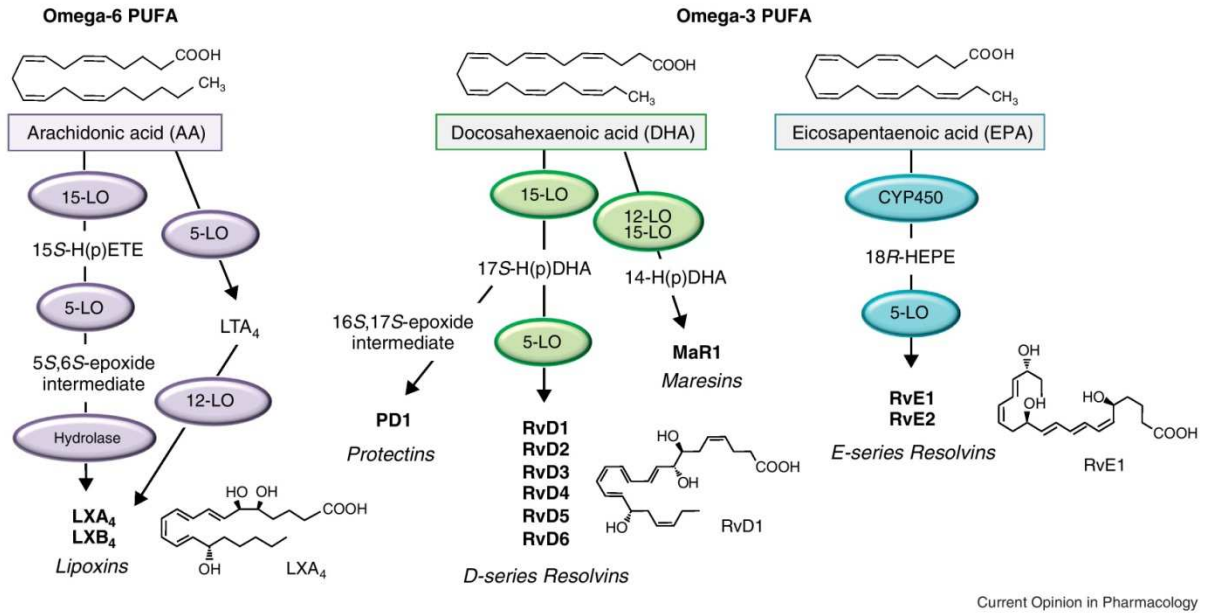

Figure 2.3: Biosynthesis of anti-inflammatory and pro-resolving lipid mediators (cited from (116)). LO, lipoxygenase; H(p)ETE, hydro(pero)xyeicosatetraenoic acid; LTA_4, leukotriene A_4; LX, lipoxin; H(p)DHA, hydro(pero)xydocosahexaenoic acid; PD1, protectin D1; RvD, resolvin D-series; RvE, resolvin E-series; MaR1, maresin 1; CYP450, cytochrome P450; HEPE, hydroxyeicosapetaenoic acid

The generation of lipoxins has been observed in mucosal tissues of the oral cavity, the respiratory and the gastrointestinal tract (137). The direct precursor 15-hydroxyeicosatetraenoic acid (15-HETE) is provided by epithelial cells whose 15-lipoxygenase acts on AA of the cell membrane (138). 15-HETE is further converted to lipoxins by 5-lipoxygenase and hydrolases in leukocytes. An alternative route includes the conversion of AA to LTA_4 in leukocytes. LTA_4 can be further metabolized to lipoxin by mucosal 15-lipoxygenase or by 12-lipoxygenase in platelets (Figure 2.3). Lipoxins act as regulators of inflammation by inhibiting the chemotaxis of neutrophils (114, 139). They reduce neutrophil and eosinophil adhesion and migration and oppose some leukocyte responses to pro-inflammatory leukotrienes. Additionally, they increase the activation of monocytes and macrophages, which take up apoptotic neutrophils. Thus, they initiate the resolution of inflammation.

The anti-inflammatory and pro-resolving actions of lipoxins and aspirin-triggered lipoxins have been demonstrated on multiple levels (reviewed in ref. (114)). In gastrointestinal physiology, lipoxin A_4 analogs were able to attenuate inflammation in chemically induced colitis in mice and even inhibit the expression of intestinal pro-inflammatory genes (140-142). Several characteristics of

inflammation during colitis could be reduced by oral treatment with lipoxin A_4 analogs including weight loss, colon injury, neutrophil infiltration and mRNA levels of several pro-inflammatory mediators like tumor necrosis factor-α and interferon-γ. A disturbed biosynthesis of lipoxin A_4 might be the reason for persistent intestinal inflammation. Colonic mucosa of patients with ulcerative colitis, a chronic inflammatory bowel disease, had significantly lower levels of lipoxin A_4 than mucosa from healthy controls (143). Only patients in medically-induced remission had an up-regulation of lipoxin A_4 levels underlining the importance of lipoxin A_4 in resolution of intestinal inflammation (144). Also in the stomach, lipoxin A_4 seems to be protective against mucosal inflammation. If the aspirin-triggered lipoxin synthesis via cyclooxygenase is blocked, the gastric-damaging actions of aspirin fully unfold (145-147). In this case, aspirin leads to increased leukocyte adhesion and, thus, promotes lesions in the stomach epithelium. Therefore, the usually observed inhibition of leukocyte adhesion by aspirin is mediated by aspirin-triggered lipoxins. Injections of lipoxin A_4 also reduced the severity of aspirin-induced gastric damage in rats (148).

A similar inhibitory effect on leukocyte adhesion is observed with resolvins. These anti-inflammatory and pro-resolving mediators are derived from the omega-3 fatty acids DHA (D-series) and EPA (E-series) (Figure 2.3). For resolvins of the E-series, EPA is converted by cytochrome P450 to 18-hydroxyeicosapentaenoic acid (18-HEPE) in endothelial cells and further transformed to resolvin E1 and resolvin E2 by 5-lipoxygenase in neutrophils (149). Both resolvins of the E-series block the transendothelial migration of neutrophils (149, 150). Resolvin E1 also enhances the clearance of apoptotic neutrophils from sites of inflammation by stimulating the phagocytic activity of macrophages (151). It influences the link to adaptive immunity by lowering the migration of dendritic cells into T cell areas of the spleen and by reducing the IL-12 production of dendritic cells (152) which lowers the generation of T helper type 1 cells (153). Resolvins have diverse sites of action. Treatment with resolvin E1 could resolve allergic airway inflammation in mice (154). The protection against developing colitis upon chemical challenge in mice proved the beneficial impact of resolvin E1 on intestinal inflammation (155-157).

Resolvins of the D-series are generated via the 15- and 5-lipoxygenase metabolism (Figure 2.3) exemplified via leukocyte-endothelial cell interactions. They are of particular interest in neural tissues, since the concentration of the precursor DHA is high in brain and synapses (158). Resolvin D1 and also its precursor 17-hydroxydocosahexaenoic acid (17-HDHA) inhibit the production of the inflammatory mediator IL-1β in microglial cells which plays a role in inflammation and host defense in neural tissues (159). But resolvins of the D-series are potent regulators of inflammation in many other locations as well. In general, resolvin D1 and D2 are potent regulators of neutrophil traffic (159-161). In the kidney, this renders resolvin D1 protective against renal ischemic injury (162). In the

intestine, resolvin D1 and D2 and their precursor 17-HDHA protect against the development of chemically induced colitis (163). Recent studies demonstrate the role of resolvins in pain regulation. In rats with adjuvant-induced arthritis, treatment with resolvin D1 and its precursor 17-HDHA reduced inflammatory pain (164, 165). Resolvin D3 has been characterized recently and its anti-inflammatory and pro-resolving activities in neutrophil regulation and macrophage stimulation has been confirmed (166). Until today, little is known about the effects of resolvin D4 to D6.

Together with resolvins, another pro-resolving lipid mediator called protectin has been described (159). Protectin is, like resolvins of the D-series, produced from DHA by neutrophils, especially in neural tissues, where it is called neuroprotectin (167). It has the same inhibitory effect on microglial cells as resolvin D1 and stimulates the phagocytosis of apoptotic neutrophils in macrophages as resolvin E1 (151, 168). Its anti-inflammatory effects include the protection against ischemic injury in the kidney (162) and in the brain (169). In humans, the association of asthma with low levels of protectin D1 has been described (170). Furthermore, the administration of protectin D1 significantly reduced symptoms of allergic airway inflammation in mice (170). Protectin D1 also links innate and adaptive immune system by directly inhibiting the cytokine production of T cells and promoting T cell apoptosis (171).

Anti-inflammatory and pro-resolving lipid mediators have manifold beneficial effects and protect against uncontrolled inflammation. They actively regulate the extent of the immune response in diverse tissues. These mediators could be the link between breast-feeding and the observed lower incidence of intestinal (172) and respiratory (9) inflammation in breast-fed infants. Human milk provides relatively high amounts of the precursors DHA, EPA and AA. But so far, it is unknown, whether maternal milk also supplies anti-inflammatory and pro-resolving lipid mediators.

2.2.3 Quantitative analysis of lipid mediators in breast milk

The only lipid mediators that have been quantified in human breast milk are prostaglandins, most often PGE_2. The mean concentrations of PGE_2 measured by radioimmunoassay vary between 149 and 902 pg/ml (173-176) which is over 100-fold higher than the concentration measured in plasma of 1.3 pg/ml (174). Concentrations of other lipid mediators, especially the anti-inflammatory ones, have not been reported yet. Although the methodology to measure lipid mediators in biological samples selectively and specifically by liquid chromatography-tandem mass spectrometry has been developed (177), it has not been applied to human milk yet.

3 Human milk oligosaccharides

The complex sugars found in human breast milk are mysterious. Next to lactose, there are more than 200 identified structures (178, 179) that are produced in the mammary gland by the concerted action of glycosyltransferase enzymes. These oligosaccharides do not have any nutritious value for the newborn, since the neonate does not possess the set of enzymes to degrade them (180). Milk oligosaccharides obviously can be absorbed, since ca. 1-2% of some ingested milk oligosaccharides have been detected in the infants' urine (181) . Absorbed milk oligosaccharides have been found to be used for the biosynthesis of gangliosides and glycoproteins (182). But for the 90% of the milk oligosaccharides that pass the small intestine unabsorbed and undigested (183), the colon lumen seems to be the site of action. The amount of oligosaccharides supplied by human milk reaches concentrations of approximately 23 g/l in colostrum and circa 13 g/l in mature milk (44, 184). Assuming an average intake for mature milk of around 700 ml per day (185), a considerable amount of several grams of oligosaccharides are passing the newborn's gut every day. They certainly must fulfill other functions than being an energy source, because the efficiency-oriented evolution would have ensured the extinction of any unnecessary usage of biosynthetic machinery.

The diversity of milk oligosaccharides is high, but the structures have certain similarities (Figure 3.1). Every milk oligosaccharide has a lactose core. This lactose core is alternatively elongated with galactose (Gal) and N-acetylglucosamine (GlcNAc) as lactosamine (Galβ1-4GlcNAc) or lacto-N-biose (Galβ1-3GlcNAc) units. The resulting oligosaccharides are linear or branched (180). Termini are usually decorated with fucose and/or N-acetylneuraminic acid (NeuAc), also called sialic acid (Sia). The presence of Sia renders oligosaccharides acidic. Milk oligosaccharides without Sia are defined as neutral. Fructo- and galactooligosaccharides are used for prebiotic supplementation of infant formula. They consist of a glucose (Glc) elongated with fructose or Gal, respectively (Figure 3.1). These artificial linear structures are very distinct to human milk oligosaccharides.

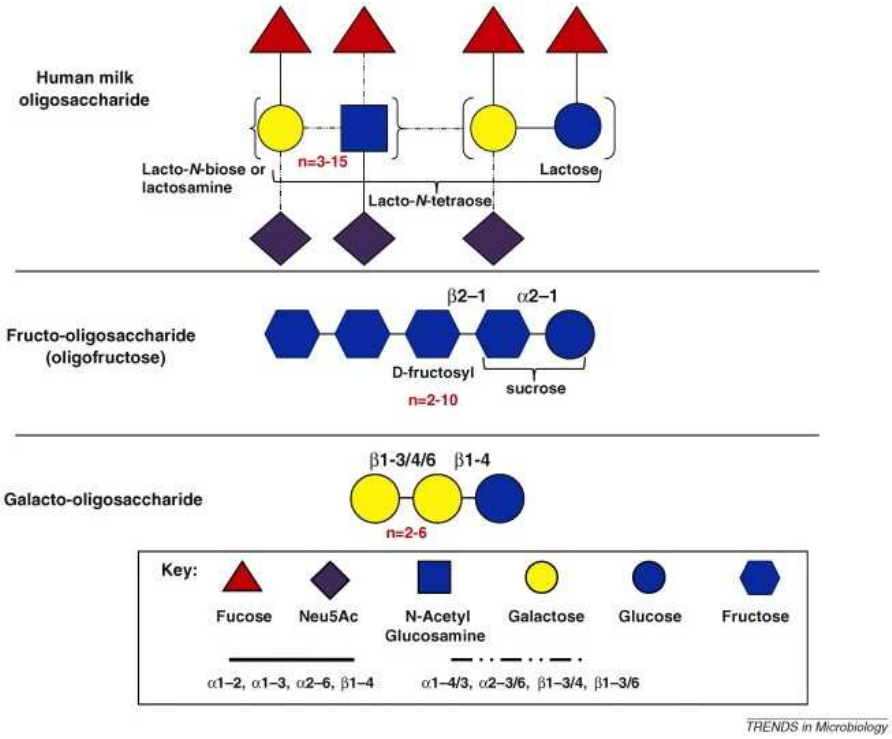

Figure 3.1: Basic structure of human milk oligosaccharides, fructo- and galactooligosaccharides (cited from (186)). Structures are depicted as symbols representing fucose (red triangle), sialic acid (N-acetylneuraminic acid, Neu5Ac, purple rhombus), N-acetylglucosamine (blue square), galactose (yellow circle), glucose (blue circle) and fructose (blue hexagon).

The glycosyltransferase enzymes responsible for the milk oligosaccharide synthesis are expressed in the mammary gland, but the exact pathway of milk oligosaccharide biosynthesis is still unknown, apart from the initial building of lactose and the final fucosylation and sialylation (187). Lactose is built by β1-4-galactosyltransferase complexed with lactalbumin that attaches Gal to Glc in the Golgi of the mammary gland epithelial cell. The required enzymes for the elongation with GlcNAc and Gal seem to be the same that are in charge for the glycosylation of proteins and lipids of the epithelial cell, namely β1,3-N-acetylglucosaminyltransferase (iGnT), β1,6-N-acetylglucosaminyltransferase (IGnT), β1,4-galactosyltransferase (β4GalT) and β1,3-galactosyltransferase (β3GalT) (188). The final decoration with fucose is done by multiple fucosyltransferases in α1-2-, α1-3- or α1-4-linkage. Sia is attached by at least three sialyltransferases in α2-3- or α2-6-linkage (187).

Depending on the linkage between Gal and GlcNAc, milk oligosaccharides are defined as type I (Galβ1-3GlcNAc; lacto-N-biose) and type II (Galβ1-4GlcNAc; lactosamine) structures (180). In humans, 2-fucosyllactose (2FL) and type I oligosaccharides containing lacto-N-biose units predominate (184). In comparison, cow's milk contains only type II milk oligosaccharides. Oligosaccharides are in general less abundant in cow's milk (189). The detected total amount of bovine milk oligosaccharides is reported to be only traces (Table 2) (45). Others found around 1 g/l in bovine colostrum rapidly

declining over two days (46, 190). Apart from type II dominance, the complexity of bovine milk oligosaccharides is lower and the grade of fucosylation and sialylation is different compared to human milk oligosaccharides (189). While approximately 70% of the human milk oligosaccharides are fucosylated, bovine milk oligosaccharides hardly contain fucose (46, 189). In cow's milk, 70% of milk oligosaccharides are sialylated (189), whereas in human milk approximately 20% contain Sia (189).

Infant formula is based on cow's milk. Because of the low concentration of milk oligosaccharides in cow's milk, the amount of oligosaccharides in non-supplemented infant formula is negligible (Table 2) (191).

Table 2: Amount of selected oligosaccharides in human breast milk and cow's milk (adapted from (45))

Component	Amount in human milk (g/l) (45)	Amount in cow's milk (g/l) (45)	Amount in bovine colostrum (g/l) (190)	Infant formula (g/l) (191)
Lactose	**55–70**	**40–50**		
Oligosaccharides				
Lacto-*N*-tetraose	0.5–1.5	Traces		
Lacto-*N*-fucopentaose I	1.2–1.7	—		
Lacto-*N*-fucopentaose II	0.3–1.0	—		
Lacto-*N*-fucopentaose III	0.01–0.2	—		
Lacto-*N*-difucohexaose I	0.1–0.2	—		
NeuAc(α2-6)lactose	0.3–0.5	0.03–0.06	0.05-0.15	0.003-0.005
NeuAc(α2-3)lactose	0.1–0.3		0.2-1.0	0.01-0.02
NeuAc-lacto-*N*-tetraose a	0.03–0.2	Traces		
NeuAc-lacto-*N*-tetraose c	0.1–0.6	Traces		
$NeuAc_2$-lacto-*N*-tetraose	0.2–0.6	Traces		
$NeuAc_2$-lactose				<0.001
NeuAc(α2-6)lactosamine	—		0.05-0.2	
Oligosaccharides (total)	**5.0–8.0**	**Traces**		

NeuAc, N-acetylneuraminic acid

Milk oligosaccharides are transferred with the breast milk into the digestive tract of the newborn where they reach the colon in their intact form. In the small intestine, they are not degraded, since the newborn does not express the required glycosidases (180). For instance, lactase cannot digest sialyl- or fucosyllactose. The neuraminidase required for cleaving off Sia and α-fucosidase for

removing fucose are not part of the brush border enzymes in humans (47). Instead of being used as an energy source, milk oligosaccharides are digested by commensal bacteria, act as soluble receptors for pathogens or influence immune processes by acting as ligands to signaling molecules. Many of these diverse functions are structure-specific that explains the variety of milk oligosaccharides.

3.1 Functions of human milk oligosaccharides

3.1.1 Prebiotic activity

The influence of breast milk on the mucosal immune response is to a large extent due to its impact on the gut microbiota. The gut microbiota is an important factor of being healthy. A disturbed composition is linked to several diseases and a disordered early colonization might increase the risk of developing them (192). The gut microbiota is established immediately after birth. Already after a few days, the amount of bacteria in the gut reaches 10^{10} colony forming units per gram feces (193, 194), already almost as much as in the adult gut which harbors approximately 10^{11} to 10^{12} bacteria per gram feces (195). But the composition of the microbiota in the adult differs greatly from the one in the neonate's gut, since the microbial composition continuously evolves during development (196). The early colonization of the intestine depends on several environmental factors. These factors comprise mode of delivery (197), the composition of the maternal microbiota (198), the mode of feeding (199), use of antibiotics, gestational age and geographical influences (192, 196, 200). The impact of the type of feeding could be to some extent due to the presence of bacteria in the milk that are able to colonize the newborn's intestine (201, 202).

As nutrition has a major impact on the bacterial composition, breastfeeding and formula-feeding result in different colonization patterns. Certainly, the content of milk oligosaccharides in human milk represents a major factor for the difference in gut colonization observed in differently fed neonates. Milk oligosaccharides are indigestible by humans. They pass the enzymatic action of the stomach and small intestine structurally unaffected and reach the colon of the infant (203, 204) where they are fermented by beneficial gut microbiota like bifidobacteria (205), and thus are able to shape the microbiota of the newborn's intestine (206). It has been shown that the predominant bacteria in breast-fed infants are bifidobacteria while the microbiological variability in formula-fed infants is more diverse (205, 207, 208). But higher levels of bifidobacteria due to breastfeeding have not been confirmed in some more recent studies (193). However, the microbiota of formula-fed infants are characterized by higher levels of less desired clostridia and *Escherichia coli* (193, 194, 207).

To utilize human milk oligosaccharides, bacteria need four types of enzymes (209). α-Fucosidase and α-sialidase are required to cleave terminal fucose and/or Sia. After this first step, the remaining structures are available for detaching GlcNAc by hexosaminidase as a monosaccharide or by lacto-N-biosidase as a disaccharide together with Gal. Gal can be detached from GlcNAc by β-galactosidase. Especially, fucosidase and sialidase activity is essential for the usage of milk oligosaccharides, since the majority of those structures are terminated by fucose (70%) or Sia (20-50%), and Gal and GlcNAc are only accessible after the removal of these terminal monosaccharides (209).

By providing specific structures that can be utilized by certain bacteria due to their enzymatic equipment, milk oligosaccharides enhance the growth of desirable bacteria like bifidobacteria and inhibit or poorly support the establishment of pathogenic bacteria like clostridia, eubacteria, enterococci or enterobacteria (199, 210). Breastfeeding leads to intestinal colonization by mainly bifidobacteria (207, 211). The proportion of bifidobacteria in the total fecal bacterial community was shown to be 60-90% in breast-fed and approximately 50% in formula-fed infants (205, 207). Bifidobacteria are described as health-promoting for the newborn and able to outcompete undesirable bacteria by creating an acidic environment which inhibits the growth of many pathogens (210, 212, 213). *Bifidobacterium longum* subsp. *infantis* (termed *Bifidobacterium infantis*) and *Bifidobacterium bifidum* employ the set of enzymes enabling using milk oligosaccharides as energy source (214, 215). In contrast, the low oligosaccharide consumer *Bifidobacterium longum* does not have the required fucosidase and sialidase enzymes (216, 217). Not all bifidobacteria can grow on human milk oligosaccharides. Strains of *Bifidobacterium adolescentis* and *Bifidobacterium animales* lack this ability (206, 218), which probably is the reason why they are not detected in the breast-fed infant gut (219).

The capability to use milk oligosaccharides is also not limited to bifidobacteria. *Bacteroides* species are as well able to grow on milk oligosaccharides (216, 220, 221). Especially, *Bacteroides fragilis* and *Bacteroides vulgatus* show strong growth on human milk oligosaccharides while species like *Clostridium difficile*, *Enterococcus faecalis*, *Escherichia coli* and *Veillonella parvula* are not able to utilize them and therefore, do not show any growth advantage (216). Bifidobacteria and bacteroides seem to have preferences when it comes to oligosaccharide consumption. Some species, e.g. *Bifidobacterium infantis*, preferentially use fucosylated oligosaccharides, while others, e.g. *Bacteroides fragilis*, favor non-fucosylated ones (216, 222). These preferences of the different bacterial strains are always dependent on the cleavage specificities of the oligosaccharide degrading glycosidases that are differently expressed by the individual strains (216). The bacterial strains employing enzymes required for degrading the lacto-N-biose unit in type 1 oligosaccharides have an advantage in colonizing the gut of the breast-fed child. The typical microbiome of the breast-fed child

probably co-developed with the predominance of type 1 oligosaccharides in human milk, since the predominant species in the breast-fed infant gut, for example *Bifidobacterium bifidum*, *Bifidobacterium breve* and *Bifidobacterium infantis*, prefer type 1 milk oligosaccharides (217, 223, 224).

Galactooligosaccharides that are often added to infant formula can also be utilized by bifidobacteria (225). A mixture of galacto- and fructooligosaccharides added to a bovine milk based infant formula changed the microbiota towards higher concentration of bifidobacteria and lower numbers of pathogens (50, 211, 226). The absolute amount and the composition of bifidobacteria in infants fed with prebiotic-supplemented formula was comparable to the one in breast-fed infants, while infants fed non-supplemented formula showed a lower absolute amount and a lower diversity of *Bifidobacterium* species (211).

The different consumption of oligosaccharides by intestinal microbiota confers a high selectivity of milk oligosaccharides in promoting bacterial growth. Human milk oligosaccharides support specific groups of beneficial bacteria, which then replace less favorable ones. Therefore, they enable the protection of the newborn against pathogens (227). Additionally, milk oligosaccharides influence the newborn's health by promoting bacteria that positively affect the maturation of the newborn's immune system (228) by modulating, for example, cytokine and antibody production (229).

3.1.2 Milk oligosaccharides as soluble receptors

Bacteria have specific glycan-binding proteins, so called lectins (230). These lectins enable host and tissue specific adhesion to the epithelial surface of the host. The adhesion involves binding to the oligosaccharides of the epithelial cell and allows the colonization of the human intestine. In case of pathogenic bacteria, adhesion is required in order to invade the host (231). Milk oligosaccharides display glyco-patterns that resemble specific binding sites for pathogens, thereby acting as soluble receptors (187). Consequently, pathogenic bacteria and also viruses can bind to milk oligosaccharides instead of the epithelial surface. The inhibited adhesion of pathogens leads to a reduction of inflammation in the intestine (183).

Anti-adhesive properties of milk oligosaccharides were shown for numerous pathogenic bacteria and viruses including *Streptococcus pneumonia* (232), *Listeria monocytogenes* (233), *Vibrio cholerae*, *Salmonella fyris*, enteropathogenic *Escherichia coli* (234) and *Campylobacter jejuni* (235) (Table 3).

Table 3: Glycan structures identified as soluble receptors for pathogens (adapted from (45, 236))

Receptor	Pathogen	Reference
Oligosaccharides	*Streptococcus pneumoniae*	(232, 237)
	Enteropathogenic *Escherichia coli* (EPEC)	(238)
	Listeria monocytogenes	(233)
	Human immunodeficiency virus (HIV)	(239)
Fucosylated oligosaccharides	*Campylobacter jejuni*	(235)
	Vibrio cholerae	(234)
	Escherichia coli (stable enterotoxin)	(240)
Fuc1-2Gal epitopes	*Candida albicans*	(241)
Sialyllactose	Cholera toxin	(242)
	Escherichia coli (S-fimbriae)	(243, 244)
	Pseudomonas aeruginosa	(245)
	Aspergillus fumigatus conidia	(246)
	Influenza virus	(45, 247, 248)
	Polyomavirus	(249)
	Helicobacter pylori	(250, 251)
Sialylated oligosaccharides	Enterotoxigenic *Escherichia coli* (ETEC) Uropathogenic *Escherichia coli* (UPEC)	(252)
Sialylated glycoproteins	*Escherichia coli* (S-fimbriae)	(253)
Sialylated glycoproteins (α2-3-linked) and sialylated poly-N-acetyllactosamine	*Mycoplasma pneumoniae*	(254)
Sialylated (α2-3)poly-N-acetylactosaminoglycans	*Streptococcus suis*	(255)
Mannosylated glycopeptide	Enterohemorrhagic *Escherichia coli* (EHEC)	(256)
Macromolecule-associated glycans	Noroviruses, *Pseudomonas aeruginosa*	(257, 258)
Gal(ß1-4)GlcNAc or Gal(ß1-3)GlcNAc	*Pseudomonas aeruginosa*	(259)
Lactadherin	Rotavirus	(260)

Milk oligosaccharides have also been shown to be protective against human immunodeficiency virus (HIV) by blocking the entry of HIV into dendritic cells (187, 239). Specific milk oligosaccharide structures inhibit the adhesion of selected bacteria due to the different binding patterns of the bacterial lectins. For instance, the S-fimbriae of *Escherichia coli* bind preferentially to sialylated structures, whereas adhesion of *Campylobacter jejuni* is inhibited by α1,2-fucosylated oligosaccharides (Table 3). Also the induction of diarrhea by the stable enterotoxin of *Escherichia coli* is inhibited by fucosylated oligosaccharides (240) at concentrations found in human milk (183). In this case, the responsible milk oligosaccharide does not bind to the pathogen, but competitively to the host cell-surface receptor which blocks the adhesion of the stable enterotoxin (261).

A recent study suggests another mechanism how milk oligosaccharides reduce the binding of pathogens (262). Exogenous milk oligosaccharides might alter the cell surface glycobiome, so that certain pathogens cannot bind anymore. The exposure to α2,3-sialyllactose (3SL) led to a decrease of Sia in the cell surface oligosaccharides and a subsequent reduction in the adhesion of

enteropathogenic *Escherichia coli* by 90% in vitro (262). The changes in the glycocalyx of the cell were due to a lower expression of genes encoding specific α2-3-sialyltransferases. This indicates that exposure to milk oligosaccharides might be able to affect gene expression.

Whichever mechanism is responsible for blocking bacterial adhesion, either binding directly to the pathogen or disabling pathogenic binding to the cell by genetically changing the glyco-pattern of the cell surface, its activation by administering oligosaccharides opens possibilities for future disease treatments (263). The manipulation of the bacterial adhesion process by carbohydrates was shown in several animal studies (reviewed in ref. (263)). For instance, the administration of lacto-N-neotetraose decreased pneumonia caused by *Streptococcus pneumoniae* in rabbits and infant rats (125). Glycopeptides reduced the attachment of *Escherichia coli* K99 to calf intestinal epithelial cells 100-fold (264). Oligosaccharides inhibit bacterial adhesion competitively. Thus, they are able to detach already bound bacteria. The oligosaccharide 3SL is able to prevent de novo adhesion, and moreover, removes bound *Helicobacter pylori* from epithelial monolayers (137).

On the basis of these in vitro and in vivo studies, carbohydrates bearing specific pathogen blocking structures are discussed as future treatment possibilities in bacterial diseases. As a result of recent advances in oligosaccharide production (265, 266), some structures are available in larger quantities. This might enable the testing of safety and efficacy by clinical trials. Studies in humans so far included the nasal administration of sialyllacto-N-neotetraose to prevent acute otitis media (267) and the oral treatment with 3SL to suppress infection with *Helicobacter pylori* (268). Both trials did not reveal a therapeutic effect suggesting a more complex interaction between bacterial lectins and epithelial cells or incorrect application and/or dosage. A proper inhibition might require not only one oligosaccharide structure, but also a mixture.

3.1.3 Regulation of immune processes by binding to proteins

Milk oligosaccharides are not only soluble receptors, but also ligands for the body's own carbohydrate-binding proteins like selectins and integrins (269). These proteins mediate cell-cell interactions of the immune system. Selectins are transmembrane proteins in the endothelial cell membrane and bind to carbohydrate moieties, for example to glycoproteins expressed on leukocytes. Thereby, they facilitate leukocyte adhesion and extravasation to sites of inflammation (48). Integrins are also cell adhesion molecules like selectins. In the form of transmembrane receptors, they are important in the signal transduction from cell to extracellular matrix and vice versa (270). They also mediate the binding of cells to diverse viruses (271).

The immune response of the human body includes the recruitment of leukocytes to sites of inflammation. But if leukocytes infiltrate epithelial cells excessively, the underlying tissues can get severely damaged (272). Leukocytes also build complexes with platelets resulting in leukocytes with an activated adhesion molecule profile and elevated production of reactive oxygen species (273). The processes of leukocyte infiltration and complex building are mediated by selectins and integrins (274). Low levels of milk oligosaccharides reach the systemic circulation and are able to bind to these carbohydrate-binding proteins since they mimic several glyco-chains found at leukocytes or pathogens (275).

The oligosaccharide structure that binds to selectins is sialyl-Lewis x, a tetrasaccharide consisting of lactosamine with a Sia attached to Gal and α1,3-linked fucose at the GlcNAc (277) (Figure 3.2). The fucosylation as well as the sialylation is required in order to bind to selectins and successfully interfere with cell-cell-interactions (48). Human milk contains several oligosaccharides that bear this selectin binding determinant (278). As selectin-ligand analogs, milk oligosaccharides regulate various immune processes (278). They inhibit leukocyte adhesion and activation as well as leukocyte-platelet complex formation, the latter of which leads to a decrease in leukocyte integrin expression (274, 279). The overall reduction of leukocyte adhesion by milk oligosaccharides causes a lower immune response of the child.

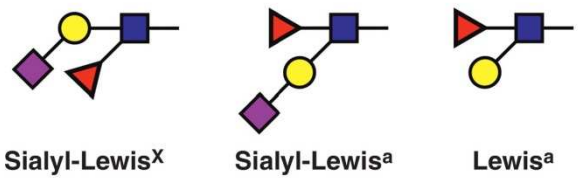

Figure 3.2: C-type lectin receptor ligands (adapted from (276)). Exemplary glycan structures that bind to C-type lections like selectins, collectins and lymphocyte lectins. Monosaccharides are depicted by symbols representing sialic acid (purple rhombus), galactose (yellow circle), N-acetylglucosamine (blue square), fucose (red triangle).

For some oligosaccharides, other direct effects on immune cells, also of the adaptive immune system, was reported. Acidic oligosaccharides isolated from human milk significantly increased the cytokine production and activation of cord blood T cells in vitro (280). Lacto-N-fucopentaose III, a milk oligosaccharide containing the trisaccharide Lewis x, was able to raise the proliferation of B cells and the production of anti-inflammatory IL-10 with the consequence of T helper cell type 1 down-regulation in mice (281). Also lacto-N-neotetraose showed the potential to increase IL-10 production in mice (282).

Not only milk oligosaccharides, but also other prebiotic oligosaccharides like galacto- and fructooligosaccharides proved to affect the immune system. Oligofructose supplementation increased the IL-10 production in Peyer's patches of the gut-associated lymphatic tissue and stimulated the cell activity of natural killer cells in rats (229). A mixture of galacto- and fructooligosaccharides, like it is used for supplementation of infant formula, influenced the

occurrence of atopic dermatitis and allergy. Administered in the first six months of life, it led to a lower incidence of atopic dermatitis over this period (283). The occurrence of allergic manifestations and infections was decreased also beyond the treatment period until the age of two years (284). As the effect is persistent also after the intake of galacto- and fructooligosaccharides, it can probably be attributed to an indirect mechanism via longer-lasting changes in the microbiota, which had been induced by the prebiotics.

These investigations demonstrate that human milk oligosaccharides and also other prebiotic oligosaccharides can influence the maturation and activation of the immune system directly, by selectin binding for example, or indirectly by increasing specific bacteria in the intestine, which themselves interact with immune cells. The immunologic functions of milk oligosaccharides are nowadays widely accepted.

3.2 Fucosyllactose

Fucosyllactose is the most abundant oligosaccharide in human milk (286). Also in this work, it received special attention and was selected for in vivo studies. It is a trisaccharide consisting of lactose with an added fucose. Fucose can be attached to milk oligosaccharides in α1-2, α1-3 and/or α1-4 linkage. In case of fucosyllactose, there are two possible structures. In 2FL, the fucose is bound to Gal in a α1-2 linkage, in 3-fucosyllactose (3FL) the fucose is attached to Glc in a α1-3-linkage. 2FL is the most abundant fucosylated milk oligosaccharide; 3FL is present in lower amounts (Figure 3.3).

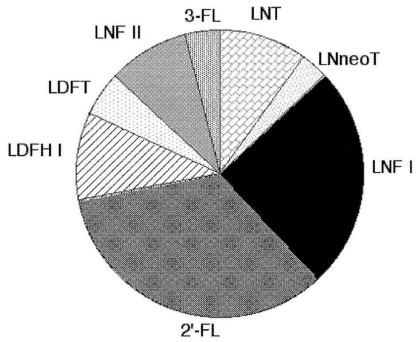

Figure 3.3: Relative abundance of fucosylated oligosaccharides in human milk (cited from (285)). LNT, lacto-N-tetraose; LNneoT, lacto-N-neo-tetraose; LNF I, lacto-N-fucopentaose I; 2'-FL, 2-fucosyllactose; LDFH I, lacto-N-difucohexaose I; LDFT, lactodifucotetraose; LNF II, lacto-N-fucopentaose II; 3-FL, 3-fucosyllactose.

The type of fucosylation occurring at the different glycostructures of the human body, like milk oligosaccharides and also blood group antigens, is genetically determined. Fucosyltransferases are expressed by two different genes. The secretor gene (*FUT2*) encodes the α1-2-fucosyltransferase and the Lewis gene (*FUT3*) family the α1-3/4-fucosyltransferase. Not every human being expresses both genes. Thus, four different groups according to their types of fucosylation can be distinguished: secretors and non-secretors who both can be either Lewis-positive or Lewis-negative. More than 20% of the human population are non-secretors (287). Women of the non-secretor type do not contain 2FL in their breast milk (288). In

combination with Lewis-negative, also other fucosylated oligosaccharides are missing. Although, these fucosylated oligosaccharides are absent, non-secretors still have an amount of milk oligosaccharides of several g/l (48). Several advantages were reported for secretors compared to non-secretors, for example reduced overall mortality and death due to NEC and sepsis in preterm infants (287).

The presence of 2-linked fucosyloligosaccharides, the typical secretor components in breast milk, is connected to several health benefits for the breast-fed newborn. Especially, its beneficial role in *Campylobacter jejuni* and norovirus infection has been thoroughly investigated. The role of 2-fucosyloligosaccharides in *Campylobacter jejuni* infection was shown in several in vitro and in vivo studies (285). On the one hand, campylobacter binding to HEp2 cells could be inhibited by 2-fucosyloligosaccharides. On the other hand, the binding could be initiated in case of Chinese hamster ovary cells that usually do not bind to *Campylobacter jejuni* by the transfection of the gene encoding human α1,2-fucosyltransferase (235). Mice were protected from campylobacter diarrhea if they were supplemented with neutral human milk oligosaccharides (235). In humans, low content of 2-linked fucosyloligosaccharides in breast milk was associated with higher occurrence of diarrhea due to *Campylobacter jejuni* and also norovirus infection in the breast-fed child (289).

In other studies, the dependency of norovirus infection on secretor status was tested (290-292). The infection with norovirus was 40 times more likely in secretors than in non-secretors (290) and milk of non-secretors was not able to inhibit the binding of norovirus to histo-blood group antigens in saliva samples (183, 257). This indicates a specific binding of norovirus to milk oligosaccharides containing α1,2-linked fucose.

The prebiotic function of 2FL and 3FL was confirmed in bacterial cultures. When human milk oligosaccharides were incubated with the entire fecal microbiome isolated from several infants, these two were among the most consumed oligosaccharides. Consumption of 2FL and 3FL led to an increase of the amount of bifidobacteria and to a reduction of the number of *Escherichia* spp. and *Clostridium perfringens* (293).

Whether fucosylated milk oligosaccharides have specific effects in gastrointestinal physiology, like sialylated milk oligosaccharides do (see 3.3), is currently unclear. In the course of this thesis, a study was conducted to answer the question if and to what extent dietary fucosyllactose influences the intestinal commensal microflora and the inflammatory response in mice. To reveal characteristics specific to the fucose linkage, 2FL as well as 3FL were included in the study.

3.3 The role of milk sialyllactose in intestinal bacterial colonization

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Running title: Sialyllactose and intestinal microbiota

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Abstract

Milk oligosaccharides influence the composition of intestinal microbiota and thereby mucosal inflammation. Some of the major milk oligosaccharides are α2,3-sialyllactose (3SL) and α2,6-sialyllactose, which are mainly produced by the sialyltransferases ST3GAL4 and ST6GAL1, respectively. Recently, we showed that mice fed milk deficient in 3SL were more resistant to dextran sulfate sodium-induced colitis. By contrast, the exposure to milk containing or deficient in 3SL had no impact on the development of mucosal leukocyte populations. Milk 3SL mainly affected the colonization of the intestine by clostridial cluster IV bacteria.

Human milk contains up to 23 g/L oligosaccharides (294). These milk oligosaccharides consist of a lactose core elongated by a variety of carbohydrates, thereby yielding more than 200 distinct structures in human milk (295).The lactose core can be extended by the attachment of galactose (Gal), N-acetylglucosamine (GlcNAc), or repeats of Gal-GlcNAc. However, the main carbohydrates added to the lactose core are fucose and the sialic acid (Sia) N-acetylneuraminic acid. Accordingly, ~70% and 28% of human milk oligosaccharides are fucosylated and sialylated, respectively (295).

Sia

The Sia family represents variants of a 9-carbon carboxylated carbohydrate backbone, which is often modified at the 4, 7, 8 and 9 positions (296). Sia occur in some bacteria (297) and vertebrates. Sia are commonly found as terminal monosaccharides in several classes of glycoconjugates, such as glycoproteins and glycosphingolipids. Gangliosides, which are a group of glycosphingolipids, are especially rich in Sia (298) and are involved in synaptogenesis and neural transmission (299). By contrast, the functional relevance of sialylated oligosaccharides in human milk is currently unknown. Because fucose and Sia are generally located at terminal positions, these carbohydrates essentially mediate the interaction of milk oligosaccharides with other molecules and cells. The effects of sialylated milk oligosaccharides have been documented in several biological assays. For example, the adhesion of leukocytes to endothelial cells can be inhibited by sialylated human milk oligosaccharides in vitro (279). Because milk oligosaccharides are partially absorbed in the intestine (300), they can occur in infants' circulation at concentrations up to 125 mg/L (279), thus possibly accounting for lower infiltration of leukocytes into intestinal tissues. Acting as structural analogs for pathogen binding receptors (301), milk oligosaccharides prevent the adhesion of pathogenic bacteria to the epithelial surface in the gastrointestinal tract. For example, α2,3-sialyllactose (3SL) binds the ulcerogenic bacteria *Helicobacter pylori*, thereby decreasing the binding of the bacteria to duodenum-derived human cells (251). Similarly, fucosylated neutral oligosaccharides that do not contain Sia also exert anti-adhesive effects. Fucosylated oligosaccharides prevent the binding of major diarrhea-causing pathogens, including *Campylobacter* and major strains of caliciviruses to epithelial cells (235, 285, 289, 302, 303).

Synthesis of sialylated oligosaccharides

The structural diversity of human milk oligosaccharides is orchestrated by multiple glycosyltransferases expressed in the lactating mammary gland. Most of these glycosyltransferases are also active in other tissues and are involved in the biosynthesis of multiple types of glycoconjugates. Accordingly, the sialyltransferases involved in the sialylation of milk

oligosaccharides are broadly expressed and have roles in various developmental and physiological pathways. Four families of sialyltransferases have been characterized, which add Sia to glycan acceptors in 3 different linkages (Table 1). A group of 6 α2,3-sialyltransferases (ST3GAL1 to 6) transfer Sia α2,3-linked to β-Gal, 2 α2,6-sialyltransferases (ST6GAL1 and 2) transfer Sia α2,6-linked to β-Gal, whereas 6 α2,6-sialyltransferases (ST6GALNAC1 to 6) transfer Sia to GalNAc (α-N-acetylgalactosamine). Finally, a group of 6 α2,8-sialyltransferases (ST8SIA1 to 6) elongate Sia through α2,8-linkage, thereby building polysialic acid chains (304-306). Such polysialic acid chains are commonly found on gangliosides and the glycoprotein neural cell adhesion molecule (N-CAM) (307), but they have never been found on milk oligosaccharides. Similarly, the enzymes of the ST6GalNAc family are mainly involved in the sialylation of O-glycan chains found on glycoproteins.

Table 1. Mammalian sialyltransferase enzymes

Family	Sialyltransferase	Principal substrates [a]	References
β-Galactoside α2,3-sialyltransferases	ST3GAL1	Galβ1,3GalNAc-(Protein)*	(308)
	ST3GAL2	Galβ1,3GalNAc-(Lipid)*	(309)
	ST3GAL3	Galβ1,3(4)GlcNAc-	(310)
	ST3GAL4	Galβ1,4(3)GlcNAc-	(310)
	ST3GAL5	Lac-Cer	(311)
	ST3GAL6	Galβ1,4GlcNAc-	(312)
β-Galactoside α2,6-sialyltransferases	ST6GAL1	Galβ1,4GlcNAc-	(313)
	ST6GAL2	Galβ1,4GlcNAc-	(314)
GalNAc α2,6-sialyltransferases	ST6GALNAC1	GalNAcα1,*O*-Ser/Thr	(315)
	ST6GALNAC2	Galβ1,3GalNAcα1,*O*-Ser/Thr	(316)
	ST6GALNAC3	NeuAcα2,3Galβ1,3GalNAc-(Lipid)*	(317)
	ST6GALNAC4	NeuAcα2,3Galβ1,3GalNAc-(Protein)*	(317)
	ST6GALNAC5	GM1b	(318, 319)
	ST6GALNAC6	GM1b, GT1b, GD1a	(320)
α2,8-Sialyltransferases	ST8SIA1	GM3	(321)
	ST8SIA2	N-glycan on N-CAM	(322)
	ST8SIA3	NeuAcα2,3Galβ1,4GlcNAc-	(323)
	ST8SIA4	N-glycan on N-CAM	(324)
	ST8SIA5	GM1b, GT1b, GD1a, GD3	(325)
	ST8SIA6	NeuAcα2,3(6)Gal-	(305)

[a] From (304)

* Preferential but not specific substrate.

The sialylation of milk oligosaccharides is mediated by members of the ST3GAL and ST6GAL families. Both ST6GAL1 and 2 enzymes are theoretically capable of catalyzing α2,6-sialylated milk

oligosaccharides. However, in contrast to ST6GAL1, the ST6GAL2 enzyme showed no activity toward lactose and lacto-N-tetraose (304) whose sialylated forms belong to the most abundant acidic oligosaccharides in human milk (326). Our data confirmed that milk 6SL (α2,6-sialyllactose) in mouse milk is generated by the action of ST6GAL1 and not ST6GAL2 (327). The levels of *ST6GAL1* transcripts were increased up to 20-fold in lactating mammary gland tissue, whereas *ST6GAL2* transcript levels were only moderately increased. Furthermore, 6SL could not be detected in milk from mice lacking the *ST6GAL1* gene (327).

The *ST6GAL1* gene is ubiquitously expressed in mammals and the corresponding enzyme acts on a broad range of glycoprotein and oligosaccharide acceptors (328). Investigations of *ST6GAL1* KO (knockout) mice demonstrated the importance of this sialyltransferase in the activation of B lymphocytes and hence in immune functions (329). In addition, ST6GAL1 affects the maturation and functionality of DC (dendritic cells) as shown by increased DC maturation in the absence of α2,6-sialylation (330). Several studies focusing on *ST6GAL1* KO mice confirmed the role of α2,6-sialylation in immunity (331-333).

In theory, several members of the ST3GAL family can account for the formation of α2,3-sialylated milk oligosaccharides. This ST3GAL family is divided into two subgroups. The first one consists of ST3GAL1 and 2. These two sialyltransferases prefer O-glycans of glycoproteins and glycosphingolipids as acceptor substrates (328). Both enzymes show a preference toward Galβ1,3GalNAc (type 3) disaccharides, but very low or no activity toward Galβ1,3GlcNAc (type 1) and Galβ1,4GlcNAc (type 2) structures found in milk oligosaccharides (309, 310). The characterization of *ST3GAL1* KO mice demonstrated the involvement of this enzyme in the sialylation of T lymphocyte proteins (334, 335).

The other ST3GAL subgroup includes the sialyltransferases ST3GAL3 to 6. ST3GAL3 and 4 prefer glycoproteins and oligosaccharides as acceptor substrates, whereas they hardly sialylate glycolipids (310). They show high activity toward type 1 and type 2 disaccharide structures. ST3GAL3 prefers type 1 over type 2 disaccharides, thus, it is responsible for the formation of the selectin ligand sialyl Lewis A on glycoproteins whose glycan backbone consists of Galβ1,3GlcNAc (type 1) disaccharides (310, 336). By contrast, ST3GAL4 prefers type 2 over type 1 disaccharides, indicating that this enzyme is involved in the biosynthesis of the sialyl Lewis X epitope on the Galβ1,4GlcNAc (type 2) backbone (336). The study of *ST3GAL4* KO mice confirmed the importance of this sialyltransferase in the formation of selectin ligands, thereby affecting leukocyte trafficking and inflammation (337-339).

The ST3GAL5 sialyltransferase synthesizes the ganglioside GM3 by transferring Sia to lactosylceramide. This sialyltransferase, also known as GM3 synthase, is not active toward other glycolipids, glycoproteins and oligosaccharides such as those found in milk (311). The ST3GAL6

enzyme preferentially transfers Sia to Galβ1,4GlcNAc (type 2) structures on glycoproteins and glycolipids (312), thereby forming, e.g., sialyl-paragloboside, which is the precursor of the sialyl Lewis X epitope on ceramide. The KO of the *ST3GAL6* gene has not yet been described in the literature.

Functional study of sialylated milk oligosaccharides

Because of the tremendous structural complexity of human milk oligosaccharides, the attribution of functions to specific milk oligosaccharides has remained a challenging task. Therefore, the functional study of milk oligosaccharides in mice has 2 advantages. First, several glycosyltransferase gene KO models are available to address the role of specific milk oligosaccharides. Second, the oligosaccharide composition of mouse milk is far less complex than that of human milk (340, 341). In mouse milk, the main oligosaccharides besides lactose are the 2 sialyllactose 6SL and 3SL and trace amounts of α1,3-fucosyllactose (341).

To study the importance of 6SL and 3SL in mouse milk, we focused on the *ST6GAL1* and *ST3GAL4* KO mouse models, because these 2 sialyltransferases are involved in the formation of 6SL and 3SL in the lactating mammary gland, respectively (327). To identify the contribution of these 2 milk oligosaccharides on mucosal immunity and physiology, we applied a cross-fostering protocol, where newborn litters of normal wildtype mice and sialyltransferase KO mice were partially exchanged at birth (Fig. 1). Along this line, newborn wild-type and KO mice were fed either normal milk or milk deficient in 3SL or 6SL. Experiments revealed no impact of 3SL or 6SL on the leukocyte population and IgA secretion in *ST6GAL1* and *ST3GAL4* KO mice and cross-fostered mice (327).

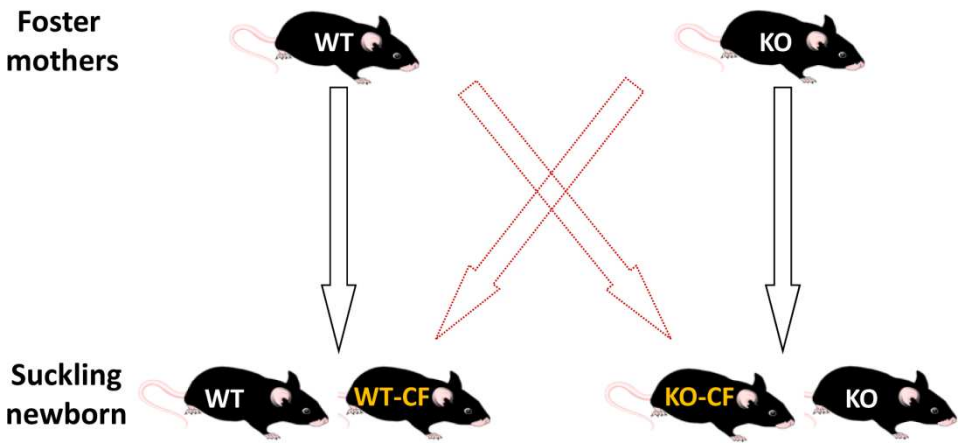

Figure 1. Schematic representation of the cross-fostering protocol between wild-type (WT) mice and sialyltransferase KO mice. Pregnancies were synchronized by time matings and newborns delivered simultaneously by WT and KO mothers were exchanged at birth. KO, knockout.

However, the investigation of 3SL and 6SL in mucosal immunity and physiology using *ST6GAL1* and *ST3GAL4* KO mice pointed to unexpected contributions of these milk oligosaccharides to the composition of intestinal microbiota. For example, analysis of 16S rDNA by real-time PCR showed that newborn mice fed 3SL-deficient milk had lower levels of clostridial cluster IV bacteria than littermates fed normal milk (327). Sequencing of 16S rDNA fragments obtained from TGGE fingerprints identified a species belonging to the family *Ruminococcaceae*, a group of clostridial cluster IV bacteria. The effect of 3SL on the colonization of the intestine may be multiple. The presence of 3SL in the milk may affect the adhesion of bacteria to sialylated structures expressed by the intestinal epithelium (342) or induce phase variation, which leads, e.g., to decreased type 1 fimbriae expression of *E. coli* (343). Specific milk oligosaccharides such as 3SL may also be used as carbon source, thereby facilitating the colonization by bacteria capable of metabolizing Sia, as shown for *E. coli*, *Vibrio cholera*, *Bacteroides fragilis*, and other pathogenic and commensal bacteria (344-346). Such a selective specificity toward 3SL supposes the expression of linkage-specific neuraminidases and transport systems by bacteria. Accordingly, clusters of NAN genes required for the catabolism of Sia are found in several pathogenic and commensal bacteria, including *Ruminococcus gnavus* (346). Thus, the ability of *Ruminococcaceae* to use Sia as carbon source could account for their enhanced growth in the presence of 3SL. Finally, sialylated milk oligosaccharides such as 3SL may influence the activation of innate immune responses by acting as ligands for pattern-recognition receptors. Along this line, Lewis X-based milk oligosaccharides have been shown to affect the binding of HIV to the DC lectin DC-SIGN (347).

The study of 3SL in *ST3GAL4* KO mice revealed that *Ruminococcaceae* were especially sensitive to the absence of 3SL in milk. The gram-positive *Ruminococcaceae* are obligate anaerobes commonly found in the intestines of mammals, including mice and humans (348). *Ruminococcaceae* are capable of assimilating polysaccharides like cellulose and starch (349-351). The genomes of several *Ruminococcus* species have been sequenced and shown to contain multiple cellulases but also several enzymes enabling the fermentation of other carbohydrates (352). *Ruminococcaceae* are enriched in patients with inflammatory bowel disease (353-355), which supports their potential role as proinflammatory bacteria.

The different microbiota composition resulting from exposure to milk containing 3SL or devoid of 3SL had a direct impact on the susceptibility of mice to acute colitis induced by dextran sulfate sodium (327). The presence of 3SL in milk decreased the resistance of mice toward dextran sulfate sodium. Several parameters such as body weight loss, colon length and epithelial permeability as well as histological examinations revealed that wild-type and *ST3GAL4* KO mice fed milk containing 3SL were more susceptible to dextran sulfate sodium-induced colitis than *ST3GAL4* KO and wild-type mice fed

3SL-deficient milk. In fact, the reconstitution of germfree mice with intestinal microbiota isolated from either wild-type or *ST3GAL4* KO mice demonstrated that these reconstituted mice showed the same susceptibility to dextran sulfate sodium as their respective microbiota donor mice. These experiments underline the importance of individual milk oligosaccharides in shaping intestinal flora and thereby in influencing the severity of intestinal inflammatory responses.

Next step: fucosylated oligosaccharides

Whereas fucosylated oligosaccharides are prominent among human milk oligosaccharides, only trace amounts of α1,3-fucosyllactose can be detected in mouse milk (341). A transgenic mouse model was developed by Prieto et al. (341), in which the human *FUT1* gene encoding the α1,2-fucosyltransferase was expressed in lactating mammary glands. Transgenic expression of human α1,2-fucosyltransferase in mice led to the biosynthesis of α1,2-fucosylated lactose in their milk. In these transgenic mice, up to 34% of the total milk oligosaccharides was accounted by α1,2-linked fucosyllactose (341).

The expression of human glycosyltransferases in mice opens new ways to study the physiological role of specific milk oligosaccharides in vivo. Similarly, murine glycosyltransferases that are not known to play a role in the milk oligosaccharide synthesis can be expressed in lactating mammary glands using transgenics technology. Accordingly, the expression of murine α1,3-galactosyltransferase in lactating mammary glands resulted in the occurrence of α1,3-galactosyllactose in mouse milk (340). Furthermore, the availability of chemically synthesized oligosaccharides in large amounts now allows testing the effect of such “new” oligosaccharides on intestinal microbiota by supplementation regimen.

The commensal bacteria of the intestine can use milk oligosaccharides as a source of energy. Especially, *Bifidobacteria* and *Bacteroides* strains are able to grow on milk oligosaccharides as the sole carbon source (356, 357). Also, non-milk oligosaccharides like fructo-, galacto-, mannan-, chito- and xylooligosaccharides can influence the intestinal microbiota. Supplementation of mice with these oligosaccharides resulted in a change of the microbiota composition in favor of *Bifidobacteria* (358, 359). Some bacterial strains like *Bifidobacterium longum* subsp. *infantis* and *Bacteroides vulgatus* show a preference for fucosylated milk oligosaccharides (356). Of note, fucosylated oligosaccharides are not present in mouse milk and *Bifidobacteria* are only minor constituents of the intestinal mouse microbiota. Supplementation of suckling mice with synthetic fucosylated oligosaccharides now allows testing the relationship between these oligosaccharides and the colonization of the mouse intestine by bacteria, such as *Bifidobacteria*.

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Abbreviations

3SL, α2,3-sialyllactose; 6SL, α2,6-sialyllactose; DC, dendritic cell; Gal, galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; KO, knockout; Sia, sialic acid

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Results

5 High levels of selected anti-inflammatory and pro-resolving lipid mediators and declining docosahexaenoic acid levels in human milk during the first month of lactation

(Manuscript submitted to *Lipids in Health and Disease*)

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5.1 Abstract

Background

The fatty acid mixture of human milk is ideal for the newborn but little is known about its composition in the first few weeks of lactation. Of special interest are the levels of long-chain PUFAs (LCPUFAs), since these are essential for the newborn's development. Additionally, the LCPUFAs arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are precursors for lipid mediators which regulate inflammation.

Methods

We determined the composition of 94 human milk samples from 30 mothers over the first month of lactation for fatty acids using GC-MS and quantified lipid mediators using HPLC-MS/MS.

Results

Over the four weeks period, DHA levels decreased, while levels of γC18:3 and αC18:3 steadily increased. Intriguingly, we found high concentrations of lipid mediators and their hydroxy fatty acid precursors in human milk, including pro-inflammatory leukotriene B4 (LTB4) and anti-inflammatory and pro-resolving lipoxin A4 (LXA4), resolvin D1 (RvD1) and resolvin E1 (RvE1). Lipid mediator levels were stable with the exception of two direct precursors.

Conclusions

Elevated levels of DHA right after birth might represent higher requirements of the newborn and the high content of anti-inflammatory and pro-resolving lipid mediators and their precursors may indicate their role in neonatal immunity and may be one of the reasons for the advantage of human milk over infant formula.

Key words

Human milk, omega-3 fatty acids, DHA, EPA, 17-HDHA, 18-HEPE, 15-HETE, resolvins, lipoxins, leukotrienes

5.2 Background

Human milk fat is the main energy source for the breast-fed newborn and provides specific fatty acids that are required for the newborn's development. The fatty acid composition of human milk has been assessed in several studies showing differences between term and preterm milk (105, 360-363) and the influence of diet on the fatty acid composition (364-366). There is also indication for a change in fatty acid composition over the lactation period (363, 367-376).

Especially, LCPUFAs fulfill several essential functions in the newborns. For example, the omega-6 LCPUFA AA and the omega-3 LCPUFA DHA are crucial for brain and nervous system development (377, 378), the visual system (379) and for early human growth in general (380). In addition, the fatty acid profile of human milk has been associated with the development of atopy and with allergic diseases in children (175, 381-383).

The supply of LCPUFAs by human milk to the newborn determines the fatty acid composition of several lipid compartments including plasma lipids and the cellular membrane (384). The fatty acid composition of the membrane influences not only membrane properties but also immune-regulatory processes through the metabolization of free and membrane bound LCPUFAs to lipid mediators (385). These lipid mediators are signaling molecules that initiate and resolve inflammation and they derive from oxygenation of the omega-6 fatty acid AA and the two omega-3 fatty acids DHA and EPA (115, 386). Oxygenation of AA, DHA and EPA occurs in a concerted action of lipoxygenases, cyclooxygenases and cytochrome-P-450 dependent oxygenases to result in the pro-inflammatory leukotrienes and the anti-inflammatory lipoxins from AA and resolvins and protectins from DHA and EPA (115, 386, 387). Apart from being anti-inflammatory, lipoxins, resolvins and protectins also initiate inflammation-resolving actions like recruitment of nonphlogistic monocytes and clearance of apoptotic polymorphonuclear neutrophils by macrophages (388-390). Due to their potent immune-regulatory functions, these lipid mediators are thought to play a role in chronic inflammatory diseases like atherosclerosis, rheumatoid arthritis and inflammatory bowel disease (387, 391-393). We therefore surmised that a supply of anti-inflammatory and pro-resolving lipid mediators to the newborn by breast milk could be one of the explanations for the lower incidence of intestinal inflammation in breast-fed compared to formula-fed infants (25, 28).

Only limited information is available on the fatty acid composition of human milk over the first few weeks of lactation (373-376), and the presence of leukotrienes, lipoxins and resolvins in human milk has not been investigated yet. In this study, we present the milk fatty acid profile and the profile for selected bioactive lipid mediators and their precursors in human milk over the first month of lactation.

5.3 Results

Quantification of fatty acid composition in human milk

Thirteen fatty acids containing 16 to 24 carbon atoms (Tab. 1 and Fig. 1) were analysed and quantified in human milk with a specific and selective GC-MS method. For each analyte, the calibration was linear with R^2>0.98 and the observed fatty acid concentrations in breast milk were within the working range (See Supplementary Tab. 1, Additional file 3). Intra-assay coefficients of variation are stated in supplementary table 1 (Additional file 3).

Table 1: Fatty acids analyzed in human milk

State of saturation	Fatty acid	Lipid number (*C*:*D*)[a]
Saturated	Palmitic acid	C16:0
	Stearic acid	C18:0
	Arachidic acid	C20:0
	Behenic acid	C22:0
	Lignoceric acid	C24:0
Monounsaturated	Palmitoleic acid	C16:1
	Oleic acid	C18:1
Omega-6 polyunsaturated	Linoleic acid	C18:2
	γ-Linolenic acid	γC18:3
	Arachidonic acid	C20:4
Omega-3 polyunsaturated	α-Linolenic acid	αC18:3
	Eicosapentaenoic acid	C20:5
	Docosahexaenoic acid	C22:6

[a] *C*, number of carbon atoms; *D*, number of double bonds

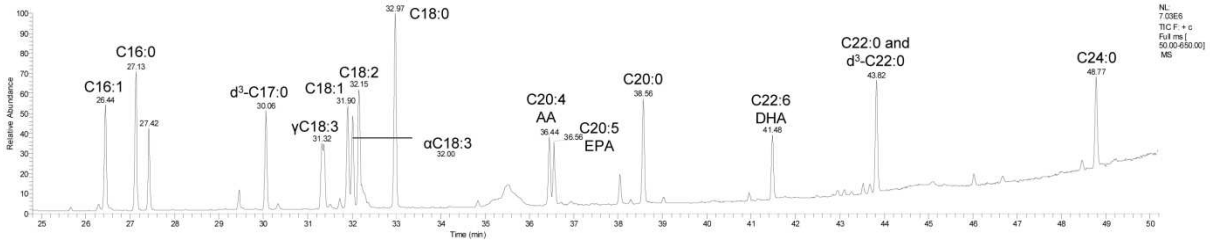

Figure 1: Gas-chromatogram of fatty acid standard substances. Fatty acids in standard mixture were methylated according to the sample preparation procedure prior to injection into the GC-MS system. For nomenclature of the fatty acids see table 1.

Fatty acid composition of human milk over the first month of lactation

The lactational age of the collected human milk samples ranged from 1 to 30 days postpartum. For statistical analysis, the samples were divided into six groups according to their lactational age (Tab. 2). The major fatty acids in human milk were C18:1, C16:0, C18:2 and C18:0 in descending order. Other fatty acids were present with quantities lower than 5% of total fatty acids. The relative content was stable over the first month of lactation for the saturated fatty acids C16:0 to C22:0. Only C24:0 showed a significant decrease (Tab. 2). The amounts of the monounsaturated fatty acids C16:1 and C18:1 were stable over the observed time period. In contrast, the amount of most PUFAs changed over time (Fig. 2). The amount of DHA decreased to approximately half of the initial quantity ($p<0.0001$, Tab. 2), while its precursor αC18:3 increased over the same period. Similarly, the amount of AA showed a trend to decrease over time ($p=0.094$) while its precursor γC18:3 increased. Albeit these changes in the concentration of the PUFAs, the ratio of omega-6 (C18:2, γC18:3, AA) to omega-3 (αC18:3, EPA, DHA) fatty acids in human milk did not change significantly over the first four weeks of lactation.

Table 2: Total fatty acid composition (%) of human milk over time of lactation

	Time of lactation (days after birth)							
	1-5 N=15	6-10 N=27	11-15 N=23	16-20 N=16	21-25 N=6	26-30 N=7		
Fatty acid	M SD	M SD	M SD	M SD	M SD	M SD	R^2	P
C16:0	23.96	20.75	21.96	21.95	21.90	21.92	0.12	0.63
	3.47	2.41	2.55	2.59	2.60	4.02		
C18:0	6.91	6.06	7.73	7.34	6.85	7.64	0.23	0.37
	1.59	1.31	1.90	1.69	0.67	1.36		
C20:0	0.17	0.11	0.15	0.14	0.12	0.12	0.36	0.10
	0.06	0.03	0.04	0.05	0.03	0.03		
C22:0	0.22	0.13	0.15	0.12	0.13	0.17	0.16	0.27
	0.18	0.04	0.06	0.04	0.04	0.03		
C24:0	0.29	0.17	0.20	0.16	0.14	0.18	0.48	0.011
	0.12	0.07	0.10	0.09	0.05	0.05		
C16:1	3.35	4.55	3.66	4.30	4.27	3.67	0.02	0.74
	0.87	1.35	1.17	1.81	1.21	0.89		
C18:1	46.29	48.21	45.76	44.92	46.26	43.96	0.46	0.51
	5.42	3.94	5.28	3.80	5.17	2.50		
C18:2	14.42	16.10	16.58	17.16	16.72	18.43	0.83	0.13
	3.09	3.24	3.97	3.37	3.45	2.80		
γC18:3	0.09	0.10	0.10	0.11	0.11	0.13	0.81	0.040
	0.03	0.02	0.04	0.04	0.02	0.02		
C20:4 (AA)	2.09	1.82	1.56	1.79	1.49	1.48	0.73	0.094
	0.96	0.91	0.62	0.83	0.41	0.49		
αC18:3	1.07	1.04	1.41	1.34	1.47	1.82	0.85	0.0024
	0.44	0.23	0.62	0.58	0.33	0.48		
C20:5 (EPA)	0.08	0.06	0.06	0.07	0.07	0.07	0.06	0.77
	0.02	0.02	0.03	0.01	0.02	0.01		
C22:6 (DHA)	1.15	0.99	0.79	0.71	0.59	0.56	0.96	<0.0001
	0.42	0.33	0.25	0.32	0.11	0.22		
n6/n3	7.58	8.92	8.31	9.47	8.81	8.27	0.12	0.59
	1.73	2.40	1.50	3.11	2.53	1.00		

M, mean % of total fatty acids; SD, standard deviation; R^2, coefficient of determination of linear trendline; P, p-value for linear trend (ANOVA).

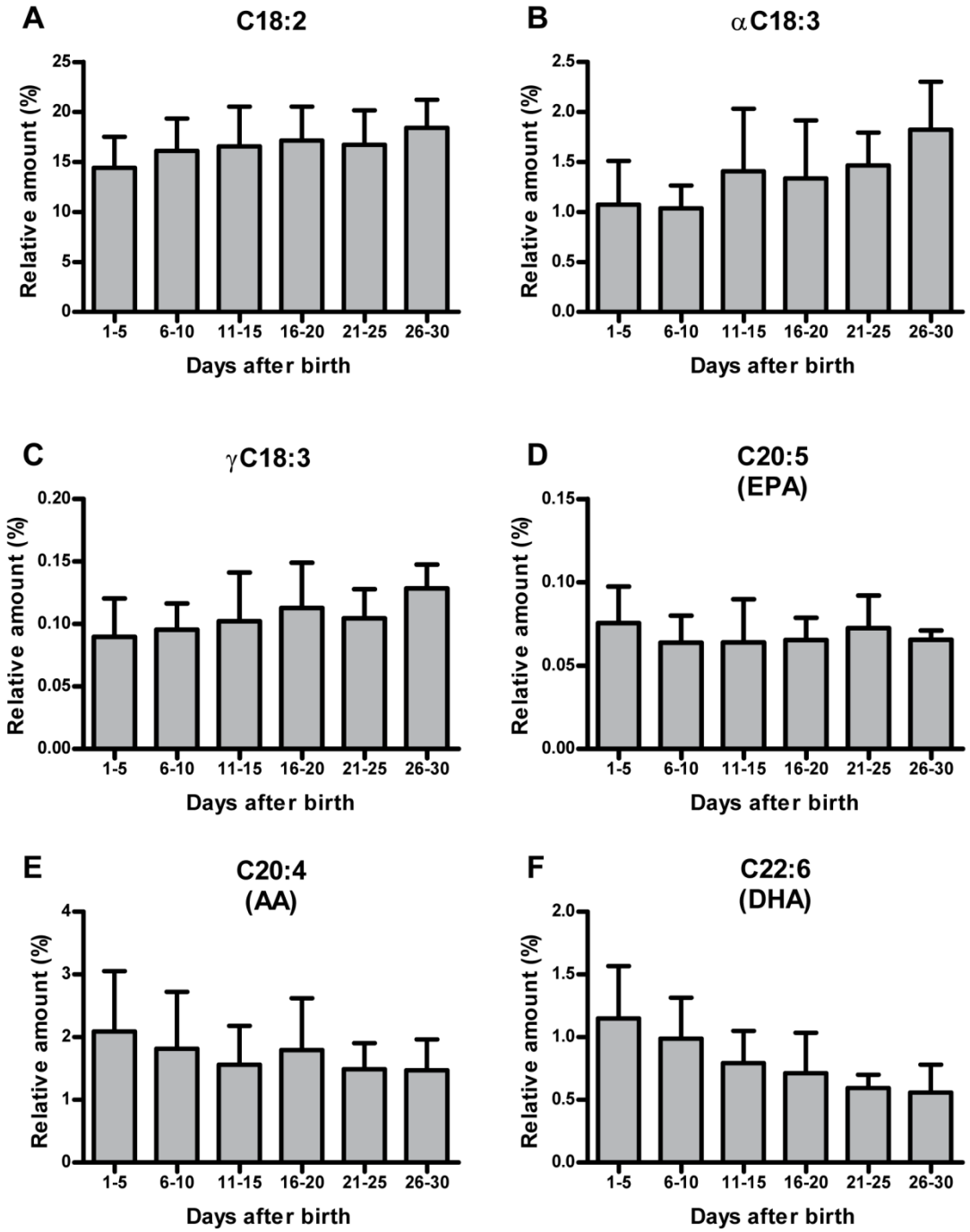

Figure 2: Changes in omega-6 and omega-3 fatty acids in human milk over the first month of lactation. Relative amount of the omega-6 PUFA (E) AA (C20:4) and its precursors (A) C18:2 and (C) γC18:3. Relative amount of the omega-3 PUFA (F) DHA (C22:6) and its precursors (D) αC18:3 and (B) EPA (C20:5). For an overview on PUFA fatty acid biosynthesis see figure 3.

Quantification of selected lipid mediators and precursors in human milk

To determine the concentration of the selected lipid mediators in human breast milk, a HPLC-MS/MS method was developed analyzing LTB4, LXA4, RvE1 and RvD1 and the four hydroxy fatty acids 17-hydroxydocosahexaenoic acid (17-HDHA), 18-hydroxyeicosapentaenoic acid (18-HEPE), 15-hydroxyeicosatetraenoic acid (15-HETE) and 12-hydroxyeicosatetraenoic acid (12-HETE) (Figs. 3 and 4). For each compound, several transitions were scanned (See Supplementary Tab. 2, Additional file 3) and one transition per analyte was selected as quantifier ion. For each lipid mediator and hydroxy fatty acid, the fragmentation pattern of the analyte from human milk was comparable to the fragmentation pattern of the corresponding standard substance (See Supplementary figure 1, Additional file 1 and Supplementary figure 2, Additional file 2). For each analyte, the calibration was linear with R^2>0.98 and the observed lipid mediator concentrations in breast milk were within the working range.

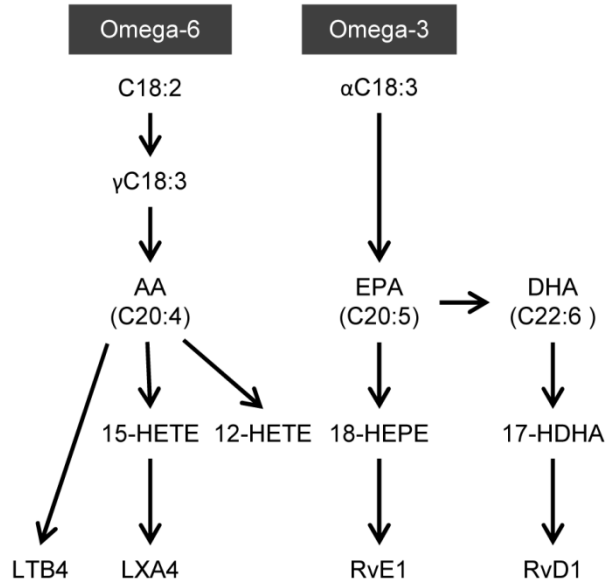

Figure 3: Simplified scheme for the biosynthesis of the selected measured lipid mediators. LTB4, LXA4, RvE1 and RvD1 are produced from the two essential omega-6 and omega-3 fatty acid pathways via the corresponding intermediate mono-hydroxy fatty acids.

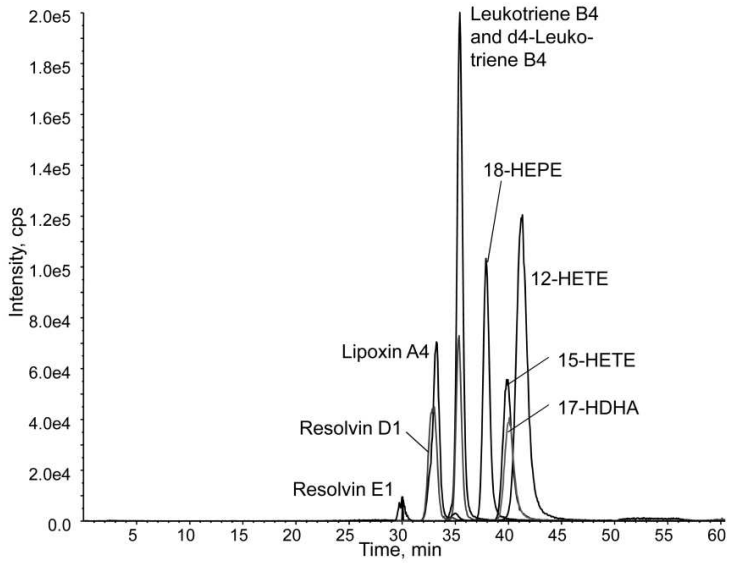

Figure 4: Chromatogram of lipid mediators and related precursor hydroxy fatty acids. 5 µl of a 0.1 ng/µl standard mixture was injected into the HPLC-MS/MS system. Cps, counts per second.

Lipid mediator concentrations of human milk over the first month of lactation

The breast milk concentration of the lipid mediators LTB4, LXA4, RvE1 and RvD1 showed mean values (and ranges) of 9 ng/ml (0.01 to 24.5 ng/ml) for LTB4, 21 ng/ml (0.1 to 54.1 ng/ml) for LXA4, 6 ng/ml (0.03 to 41.5 ng/ml) for RvE1 and 10 ng/ml (0.01 to 44.5 ng/ml) for RvD1, and was stable over the period of one month for all lipid mediators (Tab. 3). The mean concentrations of the precursor hydroxy fatty acids were higher than the concentrations of the more bioactive forms with the exception of 18-HEPE, which was observed at similar concentrations to the resulting lipid mediator RvE1 at the beginning of lactation. Over the observed four weeks of lactation, 18-HEPE increased in concentration, although not significantly (p=0.063). In contrast, the 17-HDHA content of human breast milk decreased. It was more than three times higher in the first five days postpartum than three weeks postpartum (Fig. 5), which followed the change in DHA content of the human breast milk. The concentrations of 12-HETE and 15-HETE were stable over the observed time period.

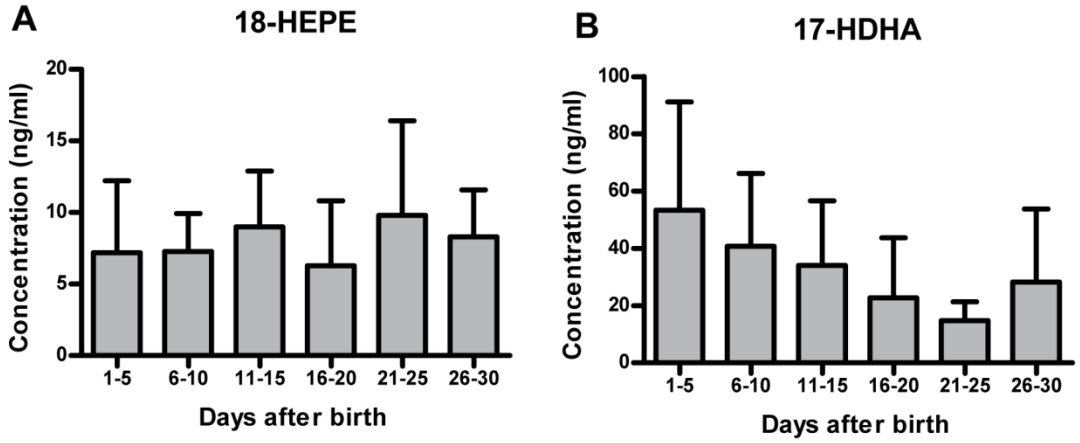

Figure 5: Changes in the concentration of the precursor hydroxy fatty acids over the first month of lactation. Concentrations of (A) 18-HEPE (precursor of RvE1) and (B) 17-HDHA (precursor of RvD1) were measured in human milk over the first month of lactation.

Table 3: Concentration (ng/ml) of lipid mediators and precursors in human milk over time of lactation

	Time of lactation (days after birth)							
	1-5 N=11-14	6-10 N=20-23	11-15 N=20-21	16-20 N=13-14	21-25 N=6	26-30 N=6-7		
Compound	M SD	M SD	M SD	M SD	M SD	M SD	R^2	P
LTB4	9.48	10.01	10.19	8.47	9.31	7.78	0.51	0.44
	8.59	4.43	5.28	6.31	5.64	3.34		
LXA4	15.55	25.02	22.11	16.65	19.53	22.04	0.02	0.19
	16.87	14.68	13.11	14.06	10.11	12.60		
RvE1	4.24	5.23	8.15	4.60	12.57	5.67	0.19	0.13
	5.76	3.91	11.45	4.08	15.20	5.65		
RvD1	9.42	12.46	12.37	6.48	9.53	9.99	0.08	0.58
	11.68	7.34	9.28	6.24	4.64	5.06		
17-HDHA	53.38	40.73	34.04	22.72	14.76	28.27	0.71	0.0035
	37.80	25.41	22.61	20.94	6.61	25.50		
18-HEPE	7.20	7.29	8.99	6.28	9.80	8.30	0.18	0.063
	5.02	2.63	3.92	4.54	6.60	3.29		
15-HETE	28.38	24.19	26.90	25.35	22.80	26.04	0.22	0.60
	16.44	12.86	11.72	17.85	8.33	19.65		
12-HETE	43.90	38.46	36.71	28.12	28.75	23.63	0.95	0.39
	26.61	29.11	15.99	19.37	15.80	11.55		

M, mean; SD, standard deviation; R^2, coefficient of determination of linear trendline; P, p-value for linear trend (ANOVA).

5.4 Discussion

In this study, we examined the fatty acid and the derived lipid mediator profiles of human breast milk over the first month of lactation. While most of the fatty acids showed stable concentrations over the four weeks of lactation, the concentrations of the omega-6 fatty acid AA and of the omega-3 fatty acid DHA decreased over time. Concomitantly, the amount of the precursor fatty acids for both of these very long-chain PUFAs increased. Human breast milk also contains a considerable amount of lipid mediators and its precursor hydroxy fatty acids. While we did not observe a change in

concentration for the lipid mediators over the investigated lactation period, the DHA metabolite 17-HDHA decreased in parallel to the DHA concentration.

The most surprising finding of this study was the high concentration of bioactive lipid mediators and their precursors in human milk. The levels of these analytes in human milk have not been reported so far. All of the investigated lipid mediators LTB4, LXA4, RvE1 and RvD1 were found at considerable concentrations, which were higher than the concentrations reported for plasma of healthy individuals. While human breast milk contains in average 8 ng/ml of the pro-inflammatory LTB4, the level for LTB4 was reported to be 100 times lower in plasma with concentrations of approximately 34 pg/ml (394). Similarly, the concentration for the anti-inflammatory lipid mediators is higher in human breast milk. While breast milk contains in average 18 ng/ml LXA4, the plasma levels were reported to be 100 times lower ranging from 10 to 26 pg/ml (395). Also, RvD1 levels are 100 times lower in plasma than in human breast milk with 31 pg/ml (135) and 9 ng/ml, respectively. The levels for RvE1 are 10 times higher in human breast milk than in plasma with 6 ng/ml and 0.1 to 0.4 ng/ml of RvE1, respectively (152). There is also a 10-100 times increase in the concentration of the precursors for these lipid mediators. While we detected 12-HETE, 15-HETE, 18-HEPE and 17-HDHA mean concentrations in human breast milk in the range of 5-40 ng/ml, these hydroxy fatty acids were measured in the low nanomolar range in human whole blood with concentrations ranging from 0.1 to 4.10 ng/ml (135, 396). Thus, there seems to be an enrichment of the lipid mediators and its precursors in human breast milk, similar to the enrichment previously observed for some of the prostaglandins (174).

Our data also suggest a preferred accumulation of anti-inflammatory and pro-resolving lipid mediators in human milk as seen by the bioactive products of AA. This fatty acid can be converted into pro- and anti-inflammatory lipid mediators like LTB4 and LXA4, respectively (397). In human milk, the average content of anti-inflammatory LXA4 is double as high as for the pro-inflammatory LTB4, while in human whole blood the concentration of LXA4 seems to be lower than the concentration of LTB4 (394, 395). In addition, there are high concentrations of the precursor hydroxy fatty acids for the anti-inflammatory and pro-resolving lipid mediators present in human breast milk. At least some of the precursors also have potent anti-inflammatory and pro-resolving effects, which may derive from a direct effect or through its metabolism to the lipid mediators.

Several studies have shown that the lipid mediators LXA4, RvE1 and RvD1 have potent anti-inflammatory and pro-resolving effects in experimental mouse models of intestinal colitis (140, 155-157, 163). In addition, also 17-HDHA the precursor for RvD1 was shown to reduce inflammation in dextran sulfate sodium induced colitis in mice (163). It is therefore tempting to speculate that these high levels of lipid mediators and their precursors in human milk might affect the immune regulation

of the gastrointestinal tract in the newborn. In this line, the concentrations measured in human breast milk for RvD1 and LXA4 meet the concentrations necessary to effect human leukocytes in an anti-inflammatory and pro-resolving manner (398, 399). For example, LXA4 and RvE1 were shown to suppress neutrophil infiltration and to promote phagocytosis of bacteria and apoptotic neutrophils by macrophages in the nanomolar range (151, 152, 388, 400).

The concentrations of the measured lipid mediators did not change over the first month of lactation in contrast to the two precursor hydroxy fatty acids 17-HDHA and 18-HEPE. While the concentration of 18-HEPE slightly increases during lactation, the concentration of 17-HDHA decreases in parallel to the decrease of its precursor fatty acid DHA. The breast milk concentrations of the lipid mediator products RvE1 and RvD1 do not show the same temporal pattern, which is in agreement with the just in time generation of these inflammatory regulators during an acute inflammation (397). Similarly, the concentrations of all the lipid mediators investigated in this study are constant in breast milk over the first month of lactation. Hence, an effect of degradation during storage can be excluded, since all samples were stored for the same time period. However, it is not clear why the intermediate precursor fatty acids investigated are present in such high concentrations in human breast milk. Possibly, these fatty acids are supplied as pre-metabolized lipid mediators for a final conversion to the more potent lipid mediators in the newborn's intestine. Considering the high concentration of these anti-inflammatory and pro-resolving hydroxy fatty acids and lipid mediators in human breast milk, we presume a potential role in lowering the overall inflammation in breast-fed infants, suggesting a possible reason for the lower incidence of inflammatory diseases in breast-fed compared to formula-fed children (401-403). In addition, these lipid mediators may be involved in gastrointestinal pain reception in newborns, since RvE1 and RvD1 have been shown to reduce inflammatory pain in mice (404).

The average fatty acid profile found in our study is similar to the fatty acid compositions reported before (360, 363, 365, 372, 381, 405). Considering the profile over the first month of lactation, the relative content in milk is stable for most fatty acids, while the concentration of specific LCPUFAs changes over this period.

Similar results were found in recent studies in which human milk with equal lactational age was analyzed and which presented the same decrease of DHA and AA over time, while the precursor fatty acids C18:2, γC18:3 and αC18:3 increased during the same period (364, 372). It was previously speculated that the increase in the precursors C18:2, γC18:3 and αC18:3 might compensate for the declining levels of DHA and AA in human breast milk, which may come from the depletion of the maternal DHA stores (87). Alternatively, the high relative amounts of DHA and AA in the beginning of lactation may well be a mechanism to compensate for the lower absolute fat content of human

breast milk in the beginning of lactation (405, 406). This may assure a stable supply of DHA and AA to the newborn suggesting a regulation of the fatty acid secretion by the mammary gland, which is not only dependent on the maternal DHA intake and plasma levels.

DHA and AA are important for the neonatal development. Especially DHA has a high abundance in the brain and retina where it is accumulated predominantly in the last trimester of pregnancy and during the first year after birth (407). Before birth, most of the accumulated DHA is delivered from the mother by transplacental transfer (408). After birth, human milk is the only exogenous source of DHA for the breast-fed child. The endogenous de novo production of the newborn and the DHA stores in adipose tissue alone are not sufficient to maintain DHA homeostasis (85). Infants fed formula without DHA showed reduced DHA stores in their tissue six months after birth and showed only half the accumulation rate for DHA in the brain compared to breast-fed infants (409). At the same time, breast-fed infants further increased their DHA stores also in non-brain tissues. Hence, the newborn's need for DHA is eminent and elevated DHA levels in human milk right after birth may represent the high requirement for DHA at that time.

Preterm infants are born with a lower DHA status, because they did not complete intrauterine DHA accumulation (410). The importance of DHA for the development is underlined by the higher DHA content of human preterm milk (105) and by the elevated activity of enzymes required for LCPUFA production in preterm infants (411). This suggests that the fatty acid composition of human milk is part of the natural mechanism to regulate the infant's DHA status.

DHA and AA are on the one hand vital for neuronal and visual functions, and on the other hand pivotal for the neonatal development of the immune system. Several studies confirmed that the DHA status is linked to various immune processes (412-416) and that several LCPUFAs have the potential to influence the immune system. For example, atopic sensitization of the newborn is associated to the α-linolenic acid and omega-3 fatty acid supply (417) and a higher tissue status of omega-3 fatty acids results in reduced inflammation and less tissue injury in a colitis model in mice (418). The latter was shown in transgenic mice expressing *fat-1*, the enzyme responsible for the endogenous production of the omega-3 and omega-6 LCPUFAs DHA and AA from the essential PUFAs. As mentioned before, these LCPUFAs are the precursors of the lipid mediators and the metabolism of these LCPUFAs to the corresponding lipid mediators may play a role in the protective effect of the LCPUFAs in innate and adaptive immune responses (419-421). Hence, our results suggest that human milk may be an important regulator of neonatal immunity by providing not only the precursors, but also the bioactive forms of these lipid mediators.

5.5 Conclusions

We investigated human milk to identify components that are responsible for the beneficial effect of human milk on neonatal health. Our results confirm a considerable amount of DHA and AA in human milk with higher levels in the beginning of lactation. Since the nutrient composition of human milk reflects the ideal mixture to satisfy the newborn's needs, this likely mirrors the neonatal DHA and AA requirements. Moreover, we demonstrate the presence of bioactive lipid mediators in human milk which affect atopy and inflammation and therefore influence neonatal immunity. Our results support an addition of DHA and AA to infant formula which is often done nowadays, but still is not standard practice. The high content of anti-inflammatory lipid mediators and its precursors in human breast milk may indicate the crucial role of lipid mediators in neonatal immunity and may be a reason for the advantage of human breast milk over infant formula.

5.6 Materials and Methods

Sample collection and preparation for fatty acid analysis

94 human milk samples were obtained from 30 mothers and the lactational age was recorded. Samples were stored at -20 °C for approximately 120 days. Sample preparation for total fatty acid analysis was essentially done according to Moser et al. (422). 125 µl of human milk was diluted 1:2 with ultrapure water (ELGA Purelab Ultra, Labtec Service AG, Wohlen, Switzerland) and subjected to protein precipitation with 1 ml methanol-methylene chloride (3:1, v/v). 20 µg d_3-C17:0 (Cambridge Isotope Laboratories, Inc., Andover, MA, USA) and 0.8 µg d_3-C22:0 (Dr. Ehrenstorfer GmbH, Augsburg, Germany) were added as internal standards. Fatty acid methyl esters were prepared by the addition of 200 µl acetyl chloride and subsequent incubation at 75 °C for one hour. The reaction was neutralized with 4 ml 7% K_2CO_3, and fatty acid methyl esters were extracted with 2 ml hexane. After centrifugation at 2500 rpm for 20 min, 1.6 ml of the hexane layer was dried under nitrogen and redissolved in 280 µl heptane for injection into the GC-MS system. Calibration curves were obtained with defatted cow's milk spiked with known concentrations of fatty acid standards, thereby defining the individual working range for each fatty acid (See Supplementary Tab. 1, Additional file 3).

GC-MS of fatty acid methyl esters

One µl sample was injected into a Finnigan PolarisQ ion trap GC-MS system (Thermo Quest, Austin, TX, USA). The injector temperature was 280 °C and fatty acid methyl esters were separated on a 30 m BGB-1701 column (BGB Analytik AG, Boeckten, Switzerland). The initial oven temperature of 50 °C

was hold for 8 min and then increased gradually by 5 °C/min reaching a final temperature of 280 °C. The ion source temperature and transfer line temperature was 230 °C and 300 °C, respectively. Analytes were detected as positive ions in full scan mode from 50 to 650 m/z. Specific mass traces were extracted for the quantification of each analyte (See Supplementary Tab. 1, Additional file 3). Fatty acids were identified by comparison of retention time and mass spectrum with authentic standards.

Sample preparation for analysis of lipid mediators and their precursors

85 of the human milk samples were stored at -20 °C for approximately 330 days. The analyte extraction method was adapted from Yang et al. (177). Two volumes of ice cold methanol and 3 ng of deuterated LTB4 (d_4-LTB4; Enzo Life Sciences AG, Lausen, Switzerland) as internal standard were added to 1 ml human milk. The samples were centrifuged for 20 min at 2500 rpm and the supernatant was diluted with 5 ml ultrapure water. The diluted supernatant was loaded on a C18 solid phase extraction (SPE) column (Grace, Deerfield, IL, USA) prewashed with 5 ml 90% methanol and 5 ml 5% methanol. After sample loading, the SPE column was washed with 2x5 ml 5% methanol and the analytes were eluted with 3 ml 90% methanol. The eluate was dried under nitrogen and redissolved in 100 µl 35% methanol (2 mM ammonium acetate). The sample was filtered with a 0.2 µm syringe filter (BGB Analytik AG, Boeckten, Switzerland) and 5 µl were injected into a HPLC-MS/MS system. Calibration curves were obtained by lipid mediator standard addition to human milk at the concentrations 0, 15, 30, 45, 60, 75 and 90 ng/ml.

HPLC-MS/MS of lipid mediators and hydroxy fatty acids

Analytes were separated using a HPLC system (UltiMate® HPLC, LC Packings, Dionex, Olten, Switzerland) with a C18 column (Luna 3u C18(2) 100A, 150x0.3 mm; Phenomenex, Brechbühler AG, Schlieren, Switzerland). A gradient was run over 10 min from 35% to 80% methanol (2 mM ammonium acetate) and was kept constant for 25 min at a flow rate of 4 µl/min. The column was rinsed with 100% methanol and equilibrated with 35% methanol (2 mM ammonium acetate). Analytes of interest were detected on a Sciex 4000 QTRAP mass spectrometer (AB SCIEX GmbH, Zug, Switzerland) in the negative ion mode. The transitions were scanned in multiple reaction monitoring mode and are stated in supplementary table 2 (Additional file 3). The optimized MS parameters were defined as: Electrospray ionization (ESI), curtain gas (CUR) = 10, nebulizer gas (GS1) = 20, auxiliary gas (GS2) = 0, ionspray voltage (IS) = -4500 V, collision gas (CAD) = medium, entrance potential (EP) = -10 V, cell exit potential = -11 to -5 V, dwell time = 100 msec. Declustering potential (DP) and collision energy (CE) were optimized individually for each analyte (See Supplementary Tab. 2, Additional file

3). Fragmentation patterns for each analyte as standard substance as well as isolated from human milk were obtained by product ion scans at MS parameter settings defined above.

Data analysis

Statistical analysis was done using Microsoft Office Excel. To estimate the difference between groups, analysis of variance (ANOVA) with subsequent trend analysis was used. All values are presented as mean with standard deviation or as indicated. Two-sided p-values < 0.05 were considered significant.

Abbreviations

12-HETE, 12-hydroxyeicosatetraenoic acid; 15-HETE, 15-hydroxyeicosatetraenoic acid; 17-HDHA, 17-hydroxydocosahexaenoic acid; 18-HEPE, 18-hydroxyeicosapentaenoic acid; AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GC-MS, gas chromatography mass spectrometry; HPLC-MS/MS, high-performance liquid chromatography tandem mass spectrometry; LCPUFA, long-chain polyunsaturated fatty acid; LTB4, leukotriene B4; LXA4, lipoxin A4; RvD1, resolvin D1; RvE1, resolvin E1; SPE, solid phase extraction

Competing interests

The authors declare that they have no competing interests.

Author's contributions

GW, DR, CB, MH designed the study and DR and CB collected the human milk samples. GW, HT and GK developed and performed the GC-MS and the LC-MS/MS assays to determine the fatty acid and lipid mediator levels in human milk. GW, HT, GK and MH analyzed the data, and GW and MH wrote the manuscript. All authors read and approved the manuscript.

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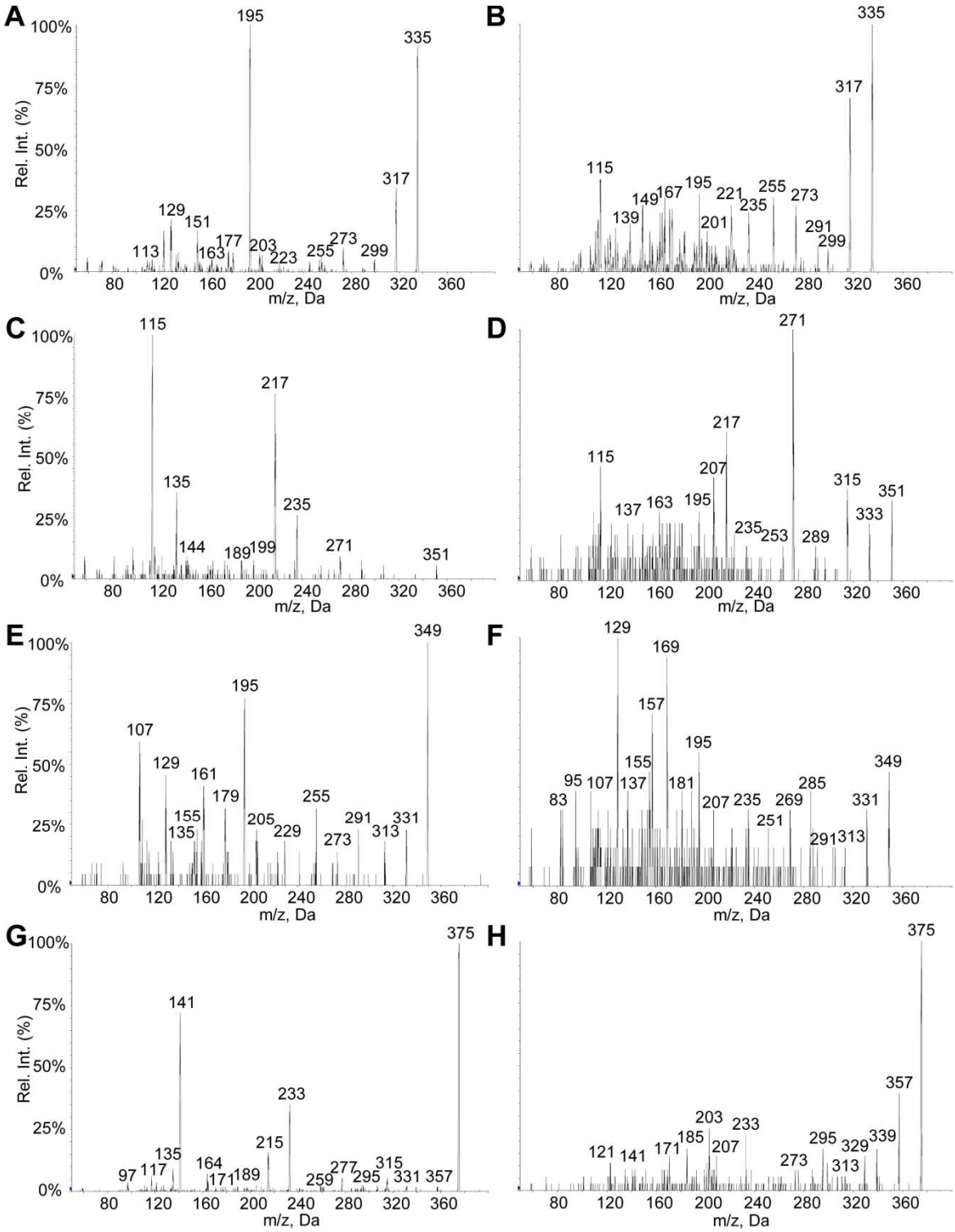
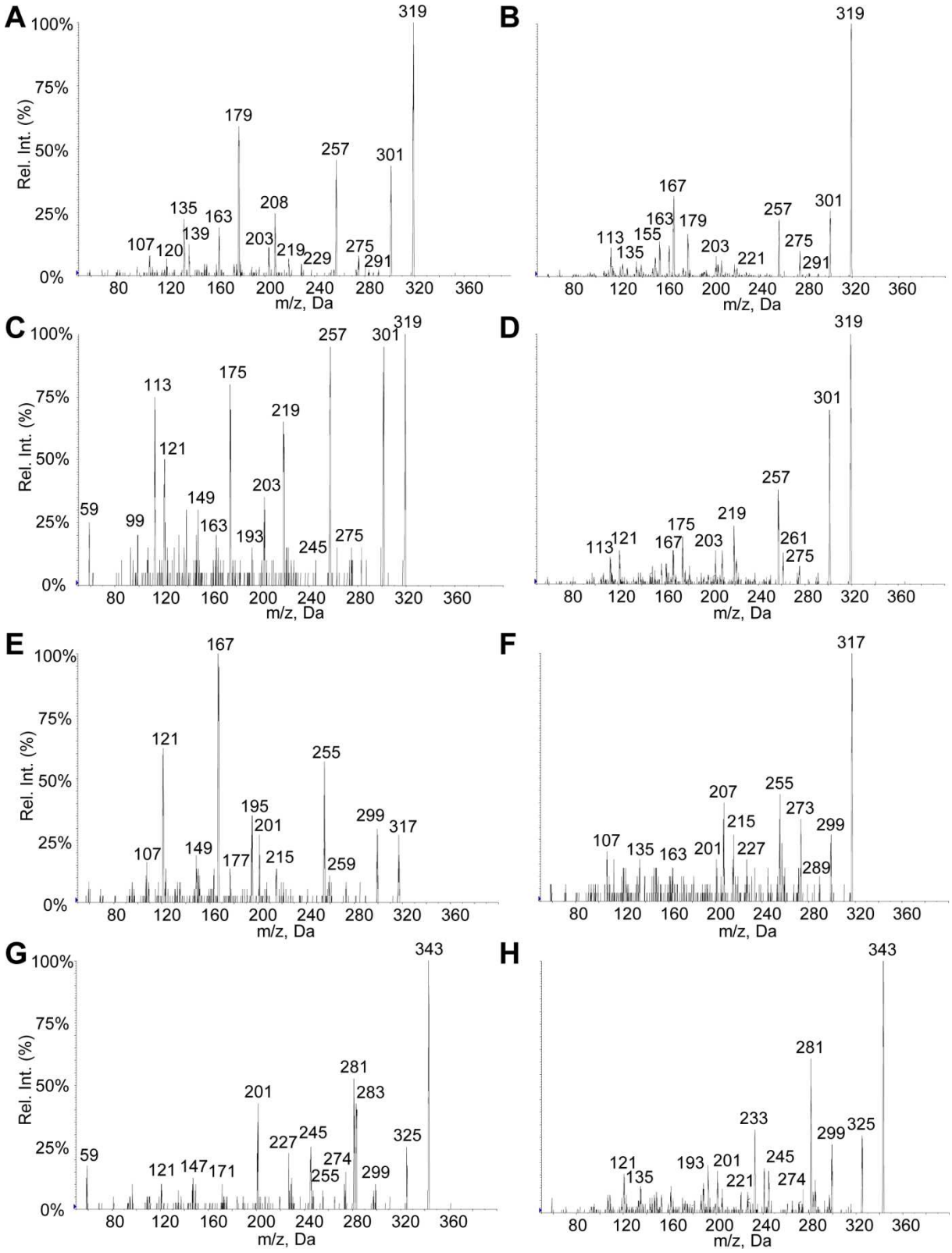
5.8 Supplemental data



Supplementary figure 1: Fragmentation spectra of lipid mediator standard substances and lipid mediators from human milk. Spectra were obtained as product ion scans at collision energy specified in Supplementary table 2, Additional file 3. Quantifier and qualifier product ions selected for each analyte are given in Supplementary table 2, Additional file 3. (A) Fragmentation of LTB4 standard and (B) LTB4 from human milk as products of 335 m/z; (C) Fragmentation of LXA4 standard and (D) LXA4 from human milk as products of 351 m/z; (E) Fragmentation of RvE1 standard and (F) RvE1 from human milk as products of 349; (G) Fragmentation of RvD1 standard and (H) RvD1 from human milk as products of 375. Rel. Int., Relative Intensity; m/z, mass per charge; Da, Dalton.



Supplementary figure 2: Fragmentation spectra of hydroxy fatty acid standard substances and hydroxy fatty acids from human milk. Spectra were obtained as product ion scans at collision energy specified in Supplementary table 2, Additional file 3. Quantifier and qualifier product ions selected for each analyte are given in Supplementary table 2, Additional file 3. (A) Fragmentation of 12-HETE standard and (B) 12-HETE from human milk as products of 319 m/z; (C) Fragmentation of 15-HETE standard and (D) 15-HETE from human milk as products of 319 m/z; (E) Fragmentation of 18-HEPE standard and (F) 18-HEPE from human milk as products of 317; (G) Fragmentation of 17-HDHA standard and (H) 17-HDHA from human milk as products of 343. Rel. Int., Relative Intensity; m/z, mass per charge; Da, Dalton.

Supplementary table I: Extracted mass traces and working range for fatty acid analysis

Fatty acid	Lipid number (*C*:*D*)[a]	Extracted mass traces (m/z)	Working range (µg/ml)	Intra-assay coefficient of variation (%) N=6
Palmitic acid	C16:0	87, 143, 270	1.2 - 2200	8.0
Stearic acid	C18:0	143, 199, 298	2.5 - 1200	12.6
Arachidic acid	C20:0	143, 283, 326	2.3 - 34	10.5
Behenic acid	C22:0	311, 354	3.5 - 60	20.2
Lignoceric acid	C24:0	143, 199, 382	3.4 - 75	16.8
Palmitoleic acid	C16:1	67, 81, 96	5.2 - 250	3.4
Oleic acid	C18:1	166, 222, 264	7.4 - 4000	2.6
Linoleic acid	C18:2	164, 220, 262	8.1 - 3000	1.9
γ-Linolenic acid	γC18:3	79, 292	0.1 - 16	12.4
Arachidonic acid	C20:4	79, 91	4.5 - 250	7.0
α-Linolenic acid	αC18:3	79, 91	1.9 - 180	15.2
Eicosapentaenoic acid	C20:5	79, 91, 384.5	1.7 - 16	12.2
Docosahexaenoic acid	C22:6	79, 91	7.1 - 120	13.0
d_3-Margaric acid	d_3-C17:0	188, 244, 287		
d_3-Behenic acid	d_3-C22:0	202, 216, 258, 314, 357		

[a] *C*, number of carbon atoms; *D*, number of double bonds

Supplementary table II: Scanned transitions for lipid mediator analysis

Compound	Scanned transition (m/z)	Collision Energy (eV)	Declustering potential (V)	Intra-assay coefficient of variation (%) N= 6 or 7
LTB4	335→317	-24	-85	6.0
	335→195 (Quantifier ion)	-24	-85	
LXA4	351→235	-28	-30	4.2
	351→217	-20	-30	
	351→115 (Quantifier ion)	-20	-30	
RvE1	349→331	-20	-30	17.5
	349→205	-24	-30	
	349→195 (Quantifier ion)	-24	-30	
	349→161	-24	-30	
RvD1	375→277	-18	-60	11.0
	375→233 (Quantifier ion)	-18	-60	
	375→215	-18	-60	
17-HDHA	343→281 (Quantifier ion)	-20	-60	9.9
	343→245	-20	-60	
	343→201	-20	-60	
18-HEPE	317→255	-25	-60	7.8
	317→195	-25	-60	
	317→167 (Quantifier ion)	-25	-60	
15-HETE	319→257	-27	-80	21.4
	319→219	-27	-80	
	319→175 (Quantifier ion)	-27	-80	
12-HETE	319→301	-23	-80	18.6
	319→257	-23	-80	
	319→179 (Quantifier ion)	-23	-80	
d_4-LTB4 (Internal Standard)	339→277	-20	-60	
	339→197 (Quantifier ion)	-20	-60	

6 Correlative association between human milk oligosaccharides and infant microbiota in humans

(Results are currently not submitted for publication)

6.1 Materials and Methods

Human milk and feces samples

Human milk was collected from one woman daily over the first three weeks of lactation to analyze changes over this period. Samples were stored at -20 °C until analysis. For studying associations between milk oligosaccharides and infant microbiota, breast milk and feces samples were provided by the University Children's Hospital Zurich and the Hospital Zollikerberg (Zurich, Switzerland) in the course of an observational clinical study (423, 424). The study protocols were approved by the Ethics Committee of the Canton of Zurich and written informed consent was obtained from all subjects. Seven women provided human milk samples at three time points after birth: 4-5 days, 9-13 days and 25-29 days. Breast milk was collected using a hospital-grade electrical breast pump. All children were delivered vaginally at term and were exclusively breast-fed. Of five corresponding children, a feces sample was collected at each collection point. Milk samples were stored at -20 °C until analysis. Feces samples were stored at -80 °C prior to 16S DNA extraction.

Sample preparation for milk oligosaccharide analysis

Human milk samples of 100 µl were centrifuged at 17000 x g for 5 min to separate milk fat. 80 µl of the medium phase were subjected to protein precipitation by addition of 9 volumes of methanol and incubation at 4 °C on ice over night. Samples were centrifuged at 17000 x g for 30 min. Supernatants were dried down and redissolved in 1 ml 2% acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA). Milk oligosaccharides were purified on a triphasic column consisting of AG 50W cation exchange resin (Bio-Rad), Sep Pak C18 SPE columns (Waters) and Supelclean™ Envi Carb™ SPE sorbent (Sigma-Aldrich). Samples were loaded after a precycle of 9 ml 2% ACN, 0.1% TFA and 6 ml 50% ACN, 0.1% TFA and conditioning with 6 ml 2% ACN, 0.1% TFA. After washing with 9 ml 2% ACN, 0.1% TFA, milk oligosaccharides were eluted with 6 ml 50% ACN, 0.1% TFA. Eluates were concentrated under nitrogen, freeze-dried and washed three times with 50 µl methanol. Purified milk oligosaccharides were redissolved in 100 µl ultrapure water and injected on a P-2 gel column (220 mm, 10 mm ID, Supelco Omnifit Column Kit) using a LaChrom HPLC (Merck Hitachi) for partial removal of lactose by

size exclusion chromatography. Ultrapure water was used as solvent at a flowrate of 0.15 ml/min. Analytes eluting from P-2 gel column in the first 70 min were collected, freeze-dried and redissolved in 50 µl ultrapure water.

Quantification of milk oligosaccharides

Purified milk oligosaccharides were separated by high-performance anion exchange chromatography (HPAEC) and detected by pulsed amperometric detection (PAD) (Dionex). Samples were diluted 1:100 and 20 µl were injected on a CarboPac PA200 column (Dionex). After an initial 5 min of 100 mM sodium hydroxide (NaOH), a linear sodium acetate (NaOAc) gradient was run over 40 min reaching 150 mM NaOAc. Flow rate was 0.35 ml/min. The column was rinsed with 1 M NaOAc, 100 mM NaOH and equilibrated with 100 mM NaOH. For peak identification, 30 sec-fractions were collected and desalted online. Milk oligosaccharides were identified by molecular mass and fragmentation pattern measured by matrix-assisted laser desorption/ionization-time of flight-tandem mass spectrometry (MALDI-TOF-MS/MS). 3SL and 6SL and lacto-N-tetraose I and lacto-N-tetraose II (lacto-N-neo-tetraose) were distinguished by comparing retention times with standard substances on the CarboPac PA200 column. Quantification was done via peak area. Amounts are given relative to peak area of lacto-N-tetraose I.

Glucose units (GU) were determined by comparing retention times with partially hydrolyzed dextran. Partial hydrolysis of dextran was done by incubation of 10 µg dextran with 100 µl 0.1 M TFA at 100 °C for 120 min, subsequent drying down, washing with methanol (4 times 50 µl) and redissoving in 100 µl ultrapure water and 10 µl were injected into the HPAEC-PAD system.

Structural identification by MALDI-TOF-MS/MS

30-sec fractions were dried down and milk oligosaccharides were labeled with 2-aminobenzamide (2-AB) by adding 20 µl of 0.35 M 2-AB, 1 M sodium cyanoborohydride ($NaCNBH_4$) in acetic acid (HAc)/dimethyl sulfoxide (DMSO) (3:7, v:v). After incubation for 120 min at 65 °C, 380 µl ACN was added and the sample was subjected to paper disk clean up on 4-7 µm filter (Whatman). Samples were loaded on filter preconditioned with 450 µl ultrapure water and 2 times 450 µl ACN. After washing with 6 times 450 µl 95% ACN, milk oligosaccharides were eluted with 3 times 50 µl ultrapure water. Eluates were freeze-dried and redissolved in 25 µl 2% ACN, 0.1% TFA. Desalting was done with HyperSep Hypercarb Columns Tips (Thermo Scientific) preconditioned with 5 times 20 µl 50% ACN, 0.1% TFA and 3 times 20 µl 2% ACN, 0.1% TFA. After sample loading, tips were washed with 10 times 20 µl 2% ACN, 0.1% TFA and milk oligosaccharides were eluted with 2 times 10 µl 50% ACN, 0.1% TFA. Samples are dried down and redissolved in 10 µl ultrapure water. 1 µl sample and 1 µl matrix

(2,5-dihydroxybenzoic acid in water-ACN (1:1, v:v), 1 mM NaCl) were spotted on a stainless steel target and recrystallized with ethanol.

MALDI-TOF-MS/MS analysis was performed on an ABI 4800 MALDI tandem time-of-flight mass spectrometer. Mass spectra were obtained in positive reflector ion mode by averaging 300 laser shots scanned across the sample surface for MS-scans and averaging scans of 1000 laser shots for MS/MS-scans. The annotation of fragments was assisted by the GlycoWorkbench tool (425).

Microbiological analysis

Total DNA was isolated from 0.1 to 0.3 g of infant feces samples using a FastDNA SPIN kit for soil (MP Biomedicals) according to the manufacturer's instructions. DNA concentrations were measured by NanoDrop (Witec AG) and 100 ng DNA was subjected to PCR. The V2-V3 region of 16S ribosomal DNA was amplified using the primers HDA1-GC 5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAGT-3' and HDA2 5'-TTACCGCGGCTGCTGGCA-3' at a temperature of 56 °C for strand annealing. After an initial denaturation at 94 °C for 4 min, 30 cycles of 30 s at 94 °C, 30 s at 56°C and 1 min at 72 °C followed. Agarose gel electrophosis was used to verify the quality of the PCR products. The separation of the amplified 16S ribosomal DNA by denaturing gradient gel electrophoresis (DGGE) and the bacterial identification by sequencing of the separated DNA was done as described in chapter 7 under materials and methods.

6.2 Results

Analysis of oligosaccharides from human milk

Oligosaccharides extracted from human milk were separated and structurally analyzed. Fourteen structures could be assigned to prominent peaks in the milk oligosaccharide chromatogram (Figure 6.1). The most prominent oligosaccharide after lactose was fucosyllactose, followed by lacto-N-fucopentaose and lacto-N-tetraose I. Lacto-N-tetraose II, lacto-N-fucoseptaose, 6SL and 3SL were present in moderate amounts. Relatively low contents were shown for lacto-N-hexaose, lacto-N-fucononaose, lacto-N-octaose, lacto-N-fucoundecaose and two sialyl-lacto-N-tetraoses. The two sialyl-lacto-N-tetraoses showed different retention times, but the Sia linkage could not be determined via fragmentation or comparison to retention times of standard substances. Thus, they were distinguished by their GU 10.3 and 10.8 determined by retention time comparison to partially

hydrolyzed dextran. The amount of lacto-N-tetraose I was selected as reference value in quantitative evaluations due to its prominence and stable amount in all analyzed human milk samples.

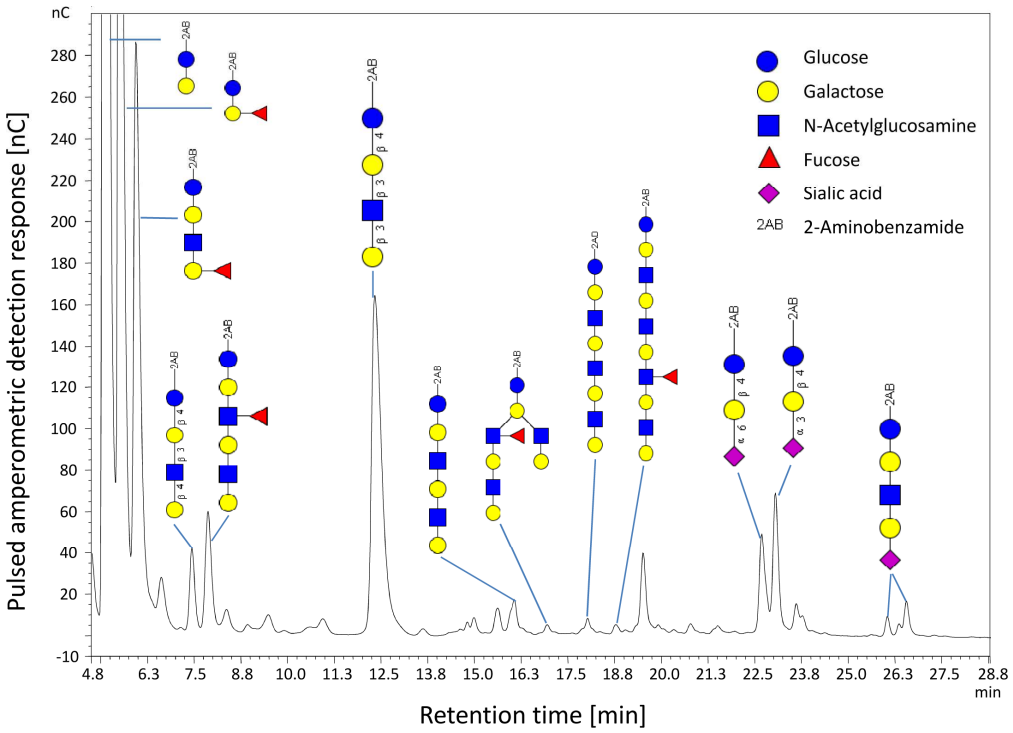


Figure 6.1: Milk oligosaccharides identified in human breast milk. Oligosaccharides extracted from human milk were analyzed by HPAEC-PAD and identified by MALDI-TOF-MS/MS.

Changes in milk oligosaccharides over lactation

The eight most prominent milk oligosaccharides were selected for quantification in milk samples collected daily from one woman over the first three weeks postpartum. Fucosyllactose could not be detected in any of the samples. As other fucosylated oligosaccharides were present, the corresponding mother seemed to be a Lewis-positive non-secretor. The composition of milk oligosaccharides changed significantly during the first two weeks after birth. Relative amounts of five of the measured milk oligosaccharides are highest directly after birth and decrease over the first two weeks postpartum (Figure 6.2A, B, D, E, F). After this initial decrease, the content of 3SL and 6SL and sialyl-lacto-N-tetraose (GU 10.3) stabilized or rose again, respectively (Figure 6.2D, E, F). In contrast, lacto-N-fucoseptaose increased in the first two weeks postpartum almost 2-fold (Figure 6.2C). The trend for sialyl-lacto-N-tetraose (GU 10.8) was unclear (Figure 6.2G).

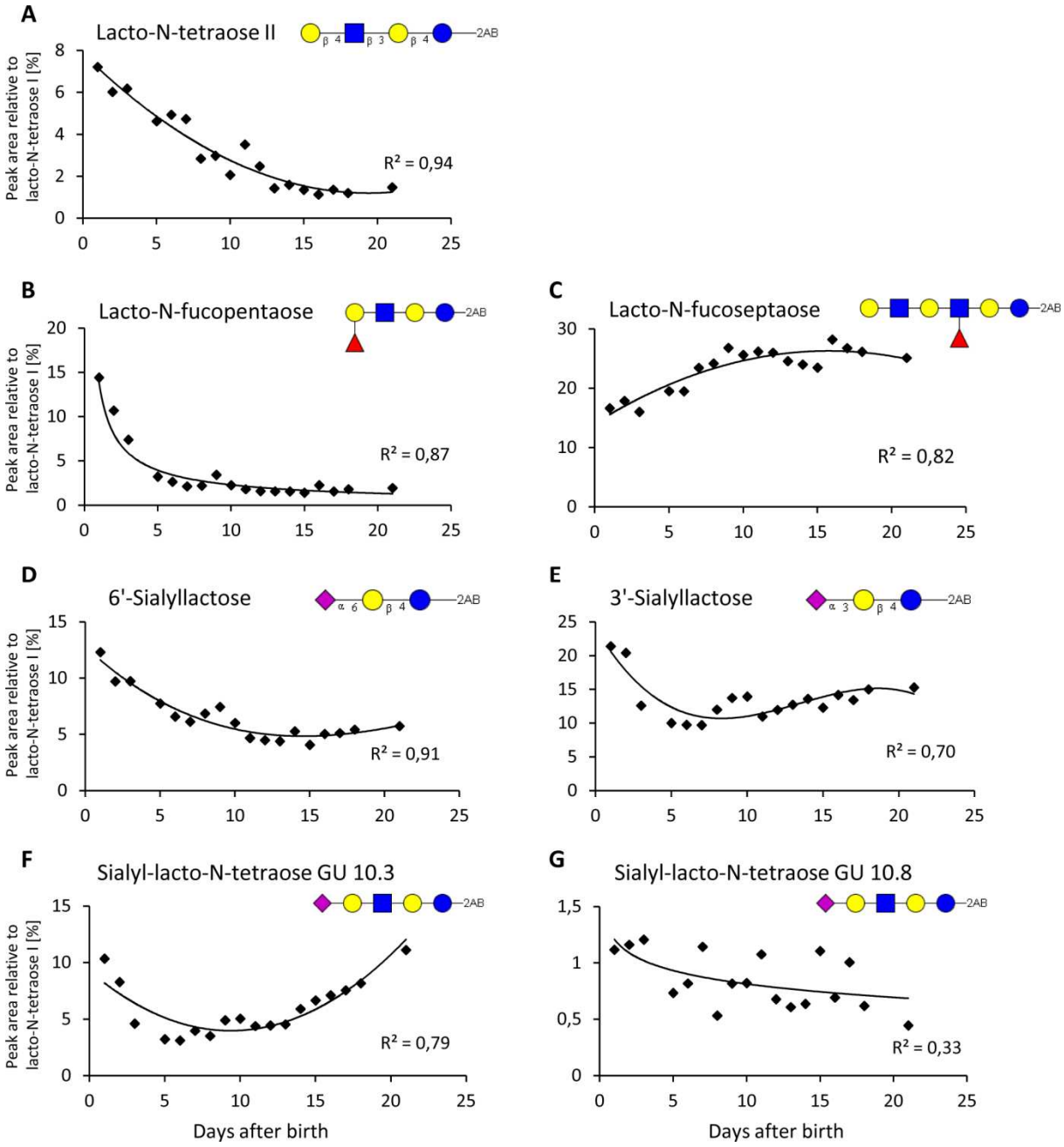


Figure 6.2: Relative amount of milk oligosaccharides over the first three weeks of lactation. Milk oligosaccharides in daily milk samples of one woman were analyzed by HPAEC-PAD. Values are given as percentages of the amount of lacto-N-tetraose I. For monosaccharide key, see Figure 7.1. GU, glucose units.

Fucosyllactose might influence the microbiota composition

Milk oligosaccharides were quantified in milk samples collected from seven different mothers at three time points during the first four weeks after birth (Figure 6.3). The previously observed decline in milk oligosaccharides over the first weeks postpartum was confirmed in the seven mothers participating in this study. For all of them, the relative amount of lacto-N-tetraose II, lacto-N-fucopentaose, 6SL and sialyl-lacto-N-tetraose (GU 10.3) decreased over the first four weeks

postpartum (Figure 6.3B, C, E, G). The relative amount of 3SL declined in human milk of four mothers, while it was stable for three women (Figure 6.3F). Lacto-N-fucoseptaose increased over the observed time period for six out of seven mothers (Figure 6.3D). Fucosyllactose and sialyl-lacto-N-tetraose (GU 10.8) showed no trend over time (Figure 6.3A, H). But for these two milk oligosaccharides, either generally low or high amounts were observed. Mothers 1, 4, 8 and 15 have more than 2-fold lower fucosyllactose concentrations than mothers 12, 16 and 18. For sialyl-lacto-N-tetraose (GU 10.8), the relative amount is approximately 2-fold higher for mothers 12, 15, 16 and 18 than for mothers 1, 4 and 8.

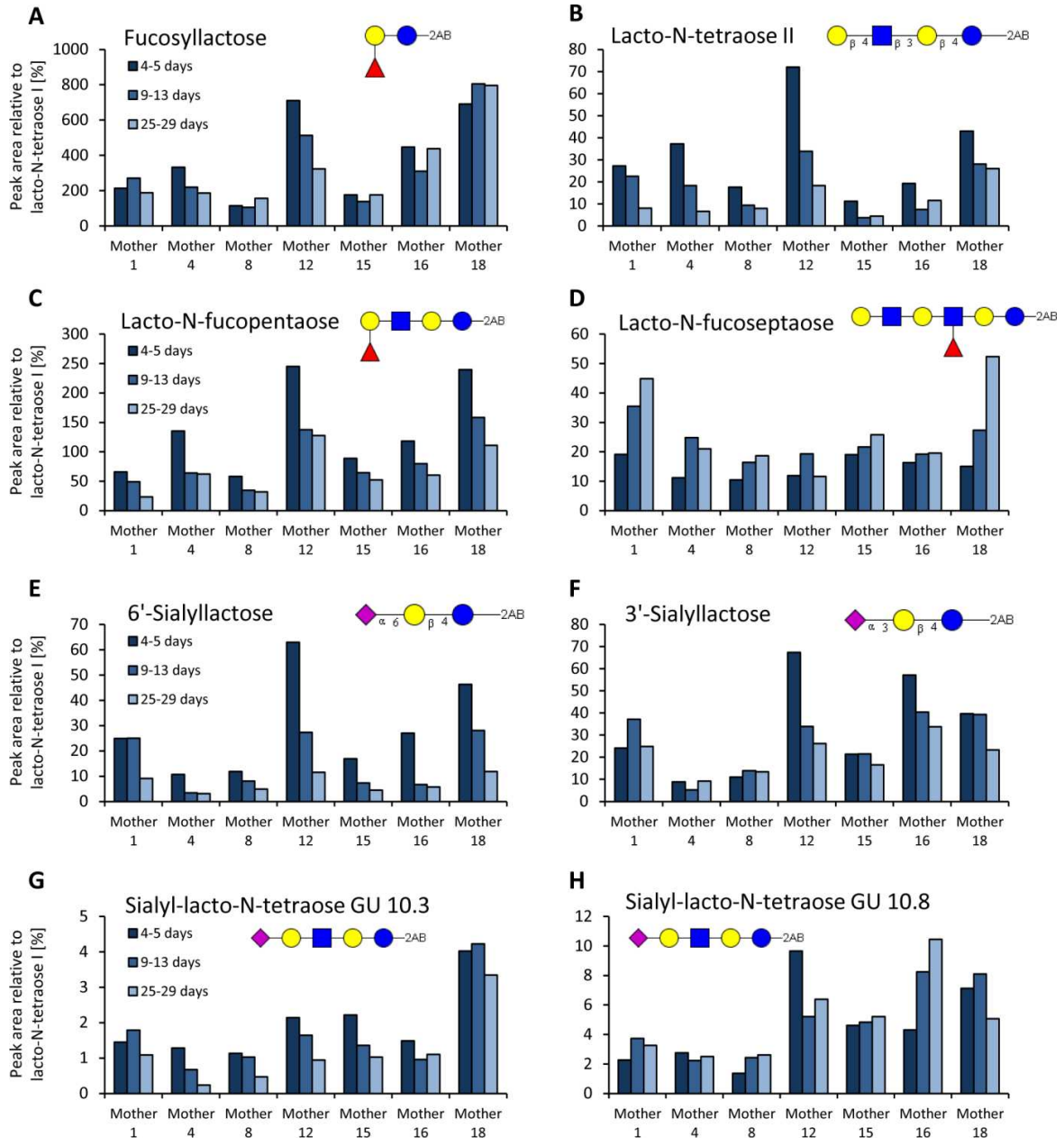


Figure 6.3: Milk oligosaccharides over the first four weeks of lactation in breast milk of seven women. Relative amounts of milk oligosaccharides were analyzed in three samples per mother at three different time points of lactation by HPAEC-PAD. For monosaccharide key, see Figure 7.1. GU, glucose units.

The variation in the infant gut microbiota was analyzed at the same time points for five (children 8, 16, 12, 15 and 18) out of seven corresponding breast-fed newborns. It was examined, if the presence of individual bacterial species in the infant feces depends on the amount of individual milk oligosaccharides in the mother's breast milk. Inter-individual differences in the amount of fucosyllactose correlated with the presence of *Bifidobacterium* spp. in the gut of the breast-fed child. In contrast to mother 8 and 15, the content of fucosyllactose in mother 16, 12 and 18 was high (Figure 6.4). *Bifidobacterium* spp. could be identified in children 16, 12 and 18, but not in 8 and 15. The presence of *Bifidobacterium* spp. seemed to be associated with the amount of fucosyllactose present in the milk.

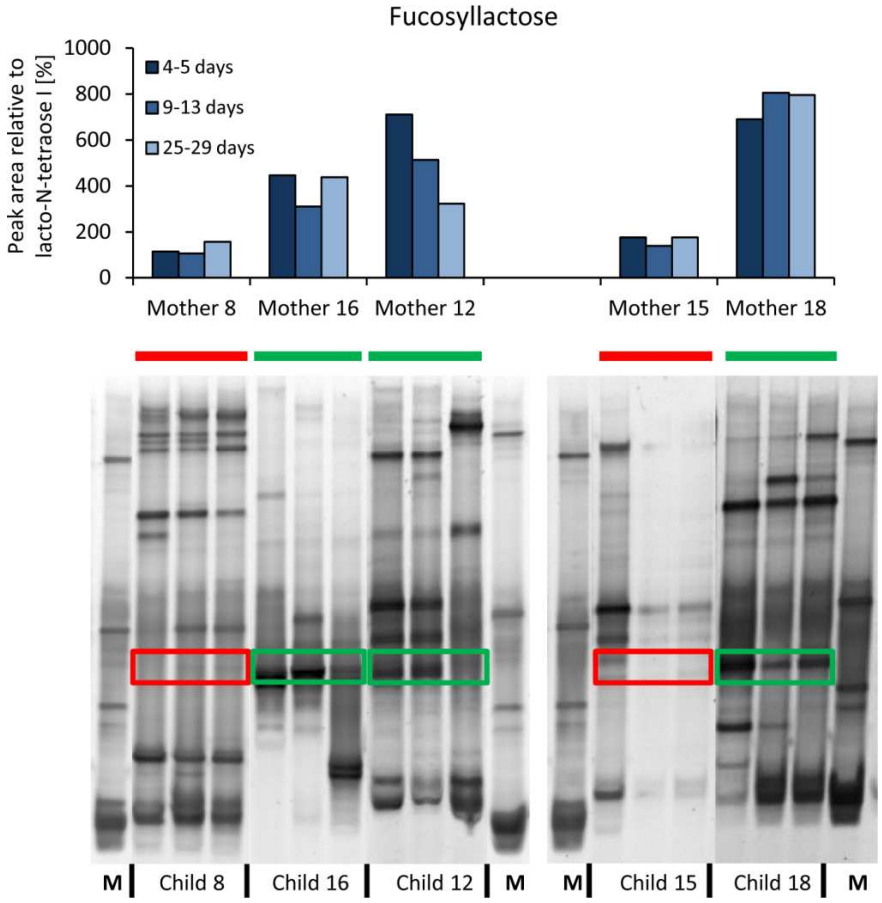


Figure 6.4: Relative amount of fucosyllactose in human milk and presence of *Bifidobacteria* spp. in infant feces. Red horizontal bars mark women with low fucosyllactose content, green bars mark women with high fucosyllactose content. 16S ribosomal DNA was isolated from infant feces and separated by DGGE. Bacterial species were identified by sequencing. Each lane represents DNA of fecal bacteria from one infant sample. The three feces samples per child are ordered by lactational age (4-6, 9-13 and 25-29 days postpartum) from left to right The band in the green boxes represents a member of the family *Bifidobacteria*. The red boxes mark children where this species is absent. M, marker.

6.3 Discussion

The composition of eight prominent oligosaccharides in human milk was observed in several mothers over three or four weeks after birth. The relative amount of most milk oligosaccharides decreased in this time period. Only lacto-N-fucoseptaose increased in concentration, whereas fucosyllactose and sialyl-lacto-N-tetraose (GU 10.8) showed rather inter-individual differences than intra-individual changes over the first month of lactation. A general decrease of neutral as well as acidic milk oligosaccharides as a whole has been reported for the first three month of lactation (286, 294, 426-428). In some studies, this decrease occurs after a concentration maximum in the first week (286, 429). While most individual structures decrease, a stable level of fucosyllactose, as seen in our study, has been shown for the first 30 days postpartum (430). Generally, the milk oligosaccharide composition and its lactational change vary greatly depending on genetic factors like expression of the secretor and Lewis genes as well as on regional factors (426, 430). However, the higher oligosaccharide concentration at the onset of lactation might support multiple aspects of the newborn's early development considering the prebiotic, pathogen- and receptor-binding activities of milk oligosaccharides.

One of the milk oligosaccharides showing great intra-individual differences was fucosyllactose, which was represented in human milk from secretors by the second largest peak after lactose. If the fucose is α1,2- or α1,3-linked could not be determined by MALDI-TOF-MS/MS. Because of its high concentration, this fucosyllactose can be assumed to represent 2FL, the usually most abundant oligosaccharide in human milk of secretors (286). This milk oligosaccharide was the only one whose concentration in human milk positively correlated with the occurrence of a bacterial species identified as member of the family *Bifidobacteria* in the feces of the corresponding infant. Aside from acting as inhibitor of intestinal pathogen-binding, e. g. of *Campylobacter jejuni* (303) and stable toxin of *Escherichia coli (289)*, 2FL has been described as important prebiotic for human microbiota (293, 431). In vitro, 2FL promotes the growth especially of bifidobacteria, as shown with the two strains *Bifidobacterium longum* and *Bifidobacterium longum* spp. *infantis* (293). The latter proved to be the one out of five tested *Bifidobacterium* species that grew best on extracted human milk oligosaccharides (357). This growth advantage might be due to an α1,2-fucosidase activity that was detected for *Bifidobacterium longum* spp. *infantis* (432) as well as *Bifidobacterium bifidum* but not for 16 other tested fecal bacteria (433). This α1,2-fucosidase activity might cause the enhanced growth of the *Bifidobacteria* species detected in our study under high levels of 2FL. The sample size of five examined mother-infant-couples does not allow drawing general conclusions about growth-promoting effects of fucosyllactose, but it demonstrates a possible association that demands further investigations in order to define the role of 2FL in the microbial colonization of the intestine.

6.4 References

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7 Selective proliferation of intestinal *Barnesiella* under fucosyllactose supplementation in mice

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Running head: Intestinal *Barnesiella* grow under fucosyllactose supplementation

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Abbreviations: 2FL, 2-fucosyllactose; 3FL, 3-fucosyllactose; DGGE, denaturing gradient gel electrophoresis; DSS, dextran sulfate sodium

7.1 Abstract

The oligosaccharides 2-fucosyllactose and 3-fucosyllactose are major constituents of human breast milk but are not found in mouse milk. Milk oligosaccharides have a prebiotic action, thus affecting the colonization of the infant intestine by microbiota. To determine the specific effect of fucosyllactose exposure on intestinal microbiota and impact on mucosal immunity in mice, we supplemented newborn mice orally with pure 2-fucosyllactose and 3-fucosyllactose. Exposure to 2-fucosyllactose and 3-fucosyllactose increased the levels of the bacterial family *Porphyromonadaceae* in the intestinal gut, more precisely members of the genus *Barnesiella* as analyzed by 16S pyrosequencing. The ability of *Barnesiella* to utilize fucosyllactose as energy source was confirmed in bacterial cultures. Whereas *Barnesiella intestinihominis* and *Barnesiella viscericola* did not grow on fucose alone, they proliferated in presence of 2-fucosyllactose and 3-fucosyllactose following the secretion of linkage-specific fucosidase enzymes that liberated lactose. The change in intestinal microbiota mediated by fucosyllactose supplementation affected the susceptibility of mice to dextran sulfate sodium-induced colitis, as shown by increased resistance of mice subjected to 2-fucosyllactose supplementation for six weeks. This study underlines the ability of specific milk oligosaccharides to change the composition of intestinal microbiota and thereby to shape an intestinal milieu resilient to inflammatory diseases.

7.2 Introduction

Oligosaccharides are a major constituent of human breast milk. Milk oligosaccharides are built up by multiple glycosyltransferases expressed in the secretory mammary gland by extending the lactose core, thereby forming close to 200 various structures (295). Milk oligosaccharides do not deliver energy to the newborn since the human gut lacks the glycosidase machinery required to break down these complex carbohydrates. Rather, milk oligosaccharides have a prebiotic action providing a nutrient milieu for the microbiotic colonization of the intestine (221), thereby influencing the development of the mucosal immune system of the breastfed infant (434). Milk oligosaccharides shape the intestinal flora by providing fermentable carbohydrates to selected bacterial groups and by acting as soluble receptors preventing the adhesion of other bacteria (252, 301, 435). The remarkable diversity of milk oligosaccharides hampers the attribution of specific functions to individual structures. To date, only few studies unraveled the biological impact of single milk oligosaccharides. For example, lacto-N-fucopentaose III was shown to induce the production of interleukin-10 and prostaglandin E2 in spleen cells (281). Disialyllacto-N-tetraose was shown to lower the incidence of necrotizing enterocolitis in rats (436). Finally, 3-sialyllactose was shown to influence the intestinal abundance of clostridial cluster IV bacteria in mice and hence, their susceptibility to dextran sulfate sodium (DSS)-induced colitis (437).

Fucosyllactose is a major constituent of human milk oligosaccharides (45), whereas only trace amounts are found in mouse milk (341). Fucosyllactose occurs as Fuc(α1-2)Gal(β1-4)Glc also called 2-fucosyllactose (2FL) and Fuc(α1-3)[Gal(β1-4)]Glc called 3-fucosyllactose (3FL). 2FL is similar to the blood group H-antigen and 3FL to the Lewis-X antigen found on glycoproteins and glycolipids (438). 2FL accounts for 20% of total human milk oligosaccharides and 3FL for less than 1% (439). The abundance of 2FL in human milk suggests an evolutionary pressure pertaining to the development of beneficial effects on infant physiology. Along this way, *ex vivo* and *in vivo* studies showed that 2FL inhibits mucosal attachment and intestinal colonization of *Campylobacter jejuni*, hence lowering the incidence of diarrhea (235, 303). Fucosyllactose also provides a protected source of lactose to bacteria expressing fucosidase enzymes, such as bifidobacteria and *Bacteroides,* whereas *Escherichia coli* and *Clostridium perfringens* cannot utilize 2FL as nutrient (440).

The potential of specific oligosaccharides to influence intestinal colonization by commensal microbiota in vivo is not known. To address the potential contribution of fucosyllactose in shaping the distribution of intestinal microbiota, we supplemented newborn mice with 2FL and 3FL, which are naturally not found in mouse milk. The lack of fucosylated oligosaccharides in mouse milk may decrease the selection of fucosidase-expressing bacteria. The exposure of infant mice to soluble 2FL

and 3FL may also impair the adhesion of bacteria to fucosylated glycans in the gastrointestinal mucosa, or interfere with the bacterial regulation of host-derived fucosylated glycans (441). Beyond the effect on intestinal microbial colonization, the question as to whether fucosyllactose supplementation has an impact on the susceptibility towards inflammatory diseases was addressed using the DSS-induced colitis model.

7.3 Results

Microbiota variation after three and six weeks of supplementation

To determine whether early exposure to exogenous fucosyllactose influenced the microbiotic colonization of the intestine, we first compared three supplementation regimens. Mice were either fed daily with 2FL or 3FL for three weeks from birth until weaning, for three weeks after weaning, or for six weeks from birth (Fig. 1). The latter protocol was referred to as long-term supplementation. Before weaning, 2FL and 3FL were given orally in addition to the natural feeding of the pups through maternal milk. The microbiological diversity in feces samples was first examined by denaturing gradient gel electrophoresis (DGGE) of amplified bacterial 16S rRNA gene fragments. By the time of weaning, the DGGE profiles obtained from 2FL-, 3FL-supplemented mice and control mice were very similar with only few DNA bands specifically found in single supplementation groups (data not shown). The main difference was represented by a band corresponding to bacteria from the family of *Lachnospiraceae* observed 2FL-supplemented mice. The DGGE profiles obtained from DNA samples isolated at six weeks of age showed more pronounced changes as exemplified by prominent bands representing members of the family of *Porphyromonadaceae* (Fig. 2). The increase in *Porphyromonadaceae* was strongest in mice supplemented with 2FL for six weeks, whereas mice supplemented with 2FL and 3FL for three weeks after weaning also showed increased *Porphyromonadaceae* yet to a lesser extent than mice exposed to 2FL or 3FL for the whole period. Because of the limited sensitivity of DGGE to quantify microbial diversity, we have also analyzed the microbial composition of DNA samples using high-throughput sequencing.

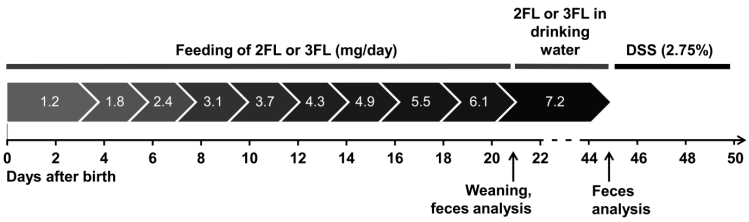

Figure 1: Fucosyllactose supplementation protocol. Fucosyllactose was fed orally until weaning with increasing daily amounts. Fucosyllactose supplementation after weaning was administered by drinking water. An average daily consumption of 4.5 ml drinking water per day and mouse resulted in a daily fucosyllactose intake of 7.2 mg. 2.75% DSS was added to drinking water from day 45 to day 50.

Each sample was analyzed at coverage ranging from 6898 to 16171 16sDNA sequences. The grouping of sequences at the level of bacterial families confirmed the results obtained by DGGE. Mice supplemented with 2FL from birth for three weeks showed increased levels of *Lachnospiraceae* (Fig. 3A) whereas other bacterial families did not vary significantly after exposure to 2FL and 3FL (Table 1).

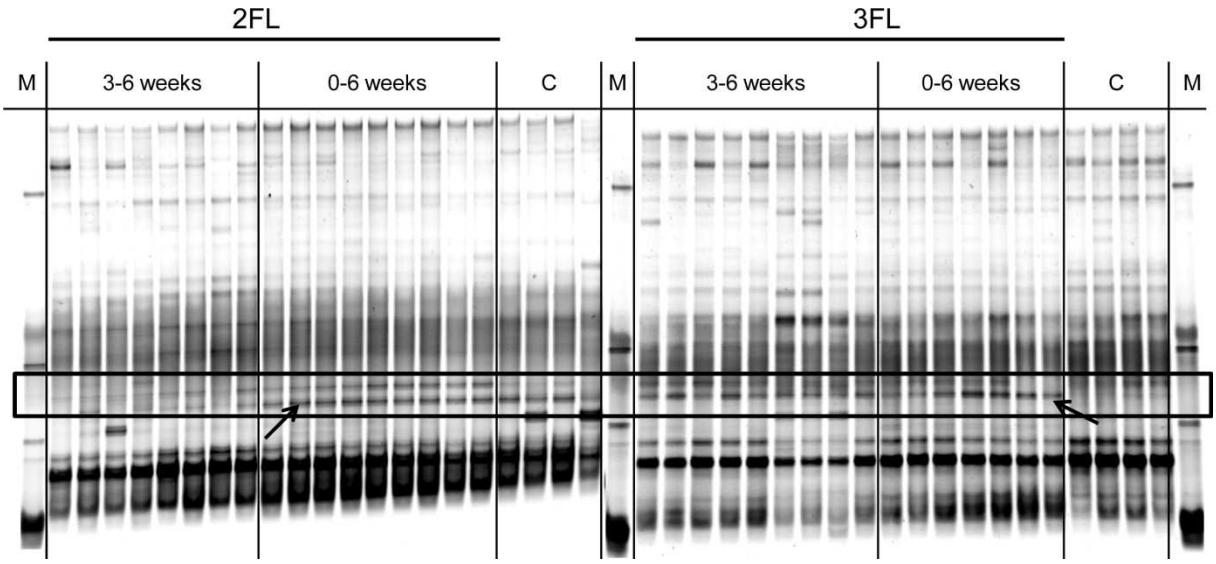

Figure 2: DGGE of bacterial 16S rDNA isolated from feces samples at age of six weeks. Bacterial variation between 2FL-, 3FL-supplemented and control mice were compared. Duration of 2FL- or 3FL- supplementation is indicated as 3-6 weeks or 0-6 weeks. Each lane represents DNA of fecal bacteria from one mouse. The lower band in the box (indicated by arrows) represents a member of the family *Porphyromonadaceae*. M, marker; C, control.

Reciprocally, the abundance of *Alcaligenaceae* strongly decreased in 2FL supplemented mice, while remaining unchanged in 3FL supplemented mice. The dominant bacterial families identified at three weeks of age were *Porphyromonadaceae* and *Bacteroidaceae*, which accounted for respectively 39% and 16% of sequence coverage in control mice. The dominance of these bacterial families in the murine gut were confirmed by Bailey et al. (442), who assigned approximately 30% of identified sequences to each of these two families. Wohlgemuth et al. also identified *Porphyromonadaceae* as the most abundant family with 37% of sequences, while they assigned 9% of sequences to *Bacteroidaceae* (443). After weaning at three weeks of age, the transition from breast milk to solid chow leads to several adjustments in the distribution of intestinal microbiota in control and fucosyllactose-supplemented mice (Table 1). The family of *Porphyromonadaceae* remained dominant in all mouse groups at six weeks of age, yet with a sharp increase in 2FL- and 3FL-supplemented mice (Fig. 3B). The analysis of bacterial sequences at the genus level showed that *Barnesiella* accounted for the observed increase in *Porphyromonadaceae* bacteria (Table 2). At the age of six weeks, varying amounts of *Barnesiella* were observed in the three mouse groups (Fig. 3C). Whereas *Barnesiella* summed up to 58.3% of identified genera in 2FL supplemented mice, *Barnesiella* reached respectively 47.0% and 39.6% of genera in 3FL supplemented mice and control mice.

Table 1: Relative abundance of bacterial families in 2FL- or 3FL-supplemented and control mice at the age of 3 and 6 weeks. Values are mean% (standard deviation). N=5 per group. Only families that are present with a mean value of more than 0.2% in at least one group are displayed.

	After 3 weeks						After 6 weeks					
	2FL		3FL		Control		2FL		3FL		Control	
Alcaligenaceae	0.5	(0.4)	6.2	(4.1)	6.9	(9.4)	2.1	(0.5)	2.2	(2.2)	1.6	(1.0)
Anaeroplasmataceae	0.0	(0.1)	2.1	(2.6)	2.7	(3.0)	3.2	(1.5)	0.9	(0.7)	3.4	(3.4)
Bacteroidaceae	27.1	(13.7)	24.4	(16.4)	15.6	(9.5)	5.2	(1.8)	7.3	(2.0)	8.4	(10.1)
Coriobacteriaceae	0.1	(0.1)	0.3	(0.2)	0.1	(0.2)	0.1	(0.0)	0.2	(0.1)	0.1	(0.1)
Deferribacteraceae	0.0	(0.0)	0.2	(0.3)	0.3	(0.3)	0.1	(0.1)	0.0	(0.0)	0.6	(0.7)
Erysipelotrichaceae	0.1	(0.1)	1.8	(1.9)	2.2	(2.8)	0.7	(0.3)	2.1	(1.2)	0.9	(1.3)
Helicobacteraceae	1.7	(1.1)	2.1	(1.9)	5.1	(3.8)	2.7	(1.0)	2.7	(0.9)	2.4	(1.2)
Incertae Sedis XIV	0.2	(0.4)	0.1	(0.1)	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
Lachnospiraceae	17.4	(8.9)	5.5	(1.4)	4.7	(3.4)	3.9	(1.0)	5.1	(2.5)	9.4	(6.2)
Lactobacillaceae	2.2	(1.0)	4.7	(4.4)	3.2	(2.3)	1.3	(1.5)	1.1	(1.4)	0.6	(0.9)
Mycoplasmataceae	0.7	(0.7)	0.2	(0.2)	0.7	(0.7)	0.4	(0.1)	0.5	(0.4)	0.1	(0.1)
Porphyromonadaceae	35.4	(9.0)	34.8	(9.9)	38.8	(8.7)	61.8	(3.8)	54.8	(4.8)	37.6	(14.9)
Prevotellaceae	5.1	(3.0)	3.2	(1.0)	4.4	(3.5)	3.7	(0.8)	3.7	(0.8)	4.9	(3.2)
Rikenellaceae	5.4	(2.1)	7.0	(4.7)	9.4	(5.6)	6.3	(0.8)	6.2	(2.9)	6.3	(2.1)
Ruminococcaceae	3.8	(1.7)	7.1	(4.0)	5.0	(2.2)	8.4	(1.6)	13.1	(3.8)	23.7	(21.1)
Verrucomicrobiaceae	0.0	(0.0)	0.0	(0.0)	0.7	(1.4)	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)

Fucosyllactose utilization by *Barnesiella*

Two species of *Barnesiella*, namely *Barnesiella viscericola* isolated from chicken caecum and *Barnesiella intestinihominis* from human faeces have been characterized and cultivated to date (444, 445). The *Barnesiella* sequences obtained in the present study showed up to 93% identity with the reference 16S rRNA sequence of *Barnesiella intestinihominis* and *Barnesiella viscericola*. The enrichment of *Barnesiella* found in fucosyllactose-supplemented mice suggested that these bacteria are able to utilize fucosyllactose as carbon source. To address this question, we have monitored the growth of *Barnesiella viscericola* and *Barnesiella intestinihominis in vitro* in culture medium containing glucose, lactose, fucose, 2FL or 3FL as sole energy source. As expected, both *Barnesiella* species grew in medium containing glucose and lactose (Fig. 4A-D). By contrast, *Barnesiella* failed to grow in presence of fucose, indicating that they cannot utilize this deoxy-hexose as carbon source (Fig. 4E, F).

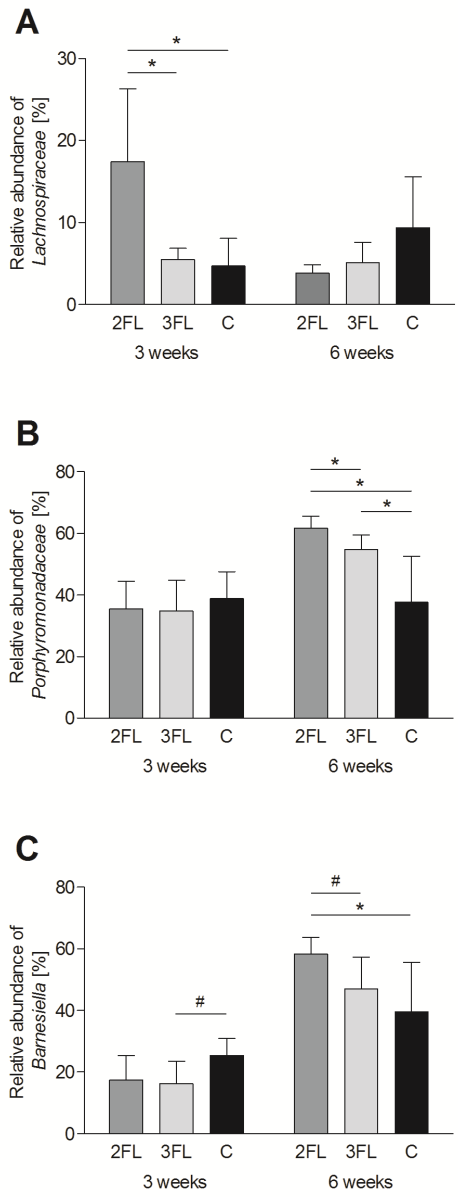

Figure 3: Individual bacterial species associated with fucosyllactose supplementation. Relative abundances of the family *Lachnospiraceae* (A), the family *Porphyromonadaceae* (B) and the genus *Barnesiella* (C) were determined in feces samples of long-term supplemented and control mice at 3 and 6 weeks of age by pyrosequencing. C, control. N = 5 per group; *, Significance with $P < 0.05$ by t-test; #, trend with $P < 0.1$ by t-test.

The addition of either 2FL or 3FL in the growth medium pointed to the differential utilization of both forms of fucosyllactose by *Barnesiella viscericola* and *Barnesiella intestinihominis*. Whereas *Barnesiella intestinihominis was* able to grow in 2FL- but not in 3FL-containing medium (Fig. 4 G, I), the reverse phenotype was observed for *Barnesiella viscericola*, which grew in presence of 3FL but not in presence of 2FL (Fig. 4H, J). Note worthily, the growth of *Barnesiella intestinihominis* in presence of 2FL was delayed, suggesting that the utilization of the oligosaccharide as energy source required the expression of a putative fucosidase. During the growth experiments, the cellular morphology of *Barnesiella* was monitored by Gram staining to confirm the purity of the two strains in culture (Supplementary fig. 1).

DSS-induced colitis in fucosyllactose supplemented mice

We finally investigated whether the changes in intestinal microbiota mediated by 2FL and 3FL supplementation affected the susceptibility of mice towards local inflammation. We therefore treated mice with DSS to induce acute colitis. Loss of body weight was assessed to monitor the severity of the inflammatory response in the different mouse groups. Supplementation of 2FL and 3FL during lactation or for three weeks after weaning had no influence on the body weight loss induced by DSS uptake (Fig. 5A, B). By contrast, 2FL supplementation for six weeks decreased the severity of DSS-induced colitis (Fig. 5C). Similarly, long-term 3FL supplementation led to reduced weight loss, although to a lesser extent than 2FL supplementation. The difference between the supplementation groups was mainly observed in male mice, which responded more strongly to DSS than females (Fig. 5D). Accordingly, male mice exposed

to 2FL and 3FL maintained higher body weight values than control mice, indicating a protective effect of the oligosaccharides towards exposure to DSS. The correlation of DSS sensitivity with the amount of *Barnesiella* bacteria in the intestine suggests that this bacterial genus confers anti-inflammatory properties in the context of DSS challenge.

Table 2: Relative abundance of bacterial genera in 2FL- or 3FL-supplemented and control mice at the age of 3 and 6 weeks. Values are mean% (standard deviation). N=5 per group. Only genera that are present with a mean value of more than 0.2% in at least one group are displayed.

	After 3 weeks						After 6 weeks					
	2FL		3FL		Control		2FL		3FL		Control	
Akkermansia	0.0	(0.0)	0.1	(0.0)	0.8	(1.7)	0.0	(0.0)	0.0	(0.1)	0.0	(0.0)
Alistipes	8.0	(3.7)	8.4	(5.8)	10.8	(7.5)	8.5	(0.9)	7.6	(3.1)	10.5	(1.8)
Allobaculum	0.0	(0.0)	0.6	(0.5)	0.0	(0.0)	0.7	(0.5)	1.6	(1.2)	0.4	(0.8)
Anaeroplasma	0.1	(0.1)	3.1	(3.8)	3.6	(4.0)	4.7	(2.1)	1.3	(1.2)	6.6	(5.6)
Bacteroides	42.5	(20.9)	34.1	(21.7)	20.2	(11.1)	7.7	(2.7)	11.2	(3.5)	15.0	(15.5)
Barnesiella	17.4	(8.0)	16.3	(7.3)	25.4	(5.4)	58.3	(5.4)	47.0	(10.2)	39.6	(16.1)
Blautia	0.4	(0.7)	0.1	(0.2)	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
Butyricicoccus	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)	0.2	(0.2)	0.0	(0.1)	0.4	(0.6)
Coprobacillus	0.1	(0.1)	0.7	(0.9)	1.4	(1.5)	0.2	(0.1)	0.5	(0.4)	0.4	(0.4)
Helicobacter	2.5	(1.7)	2.8	(2.4)	6.1	(4.8)	3.7	(1.4)	3.8	(1.3)	4.4	(3.5)
Lactobacillus	3.6	(2.1)	7.0	(6.9)	4.4	(3.2)	1.9	(2.1)	1.8	(2.4)	1.1	(1.5)
Marvinbryantia	0.0	(0.0)	0.1	(0.1)	0.0	(0.0)	0.1	(0.0)	0.1	(0.1)	0.3	(0.2)
Mucispirillum	0.0	(0.0)	0.4	(0.4)	0.4	(0.4)	0.1	(0.1)	0.0	(0.1)	1.7	(2.2)
Odoribacter	0.2	(0.1)	0.0	(0.0)	0.8	(0.8)	0.6	(0.2)	0.0	(0.0)	0.9	(0.5)
Oscillibacter	2.3	(1.1)	1.1	(1.0)	1.1	(0.9)	1.8	(0.4)	1.9	(1.1)	3.6	(2.6)
Parabacteroides	13.9	(6.4)	13.1	(4.9)	11.4	(6.5)	4.1	(2.2)	13.3	(7.5)	5.0	(6.4)
Paraprevotella	0.0	(0.0)	0.0	(0.0)	0.3	(0.6)	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
Parasutterella	0.4	(0.4)	6.3	(4.2)	7.3	(10.0)	1.6	(0.3)	2.7	(2.9)	2.7	(1.6)
Prevotella	6.1	(3.4)	3.6	(1.1)	3.9	(2.3)	4.2	(0.9)	4.4	(1.0)	5.3	(1.9)
Roseburia	0.1	(0.1)	0.7	(0.6)	0.1	(0.2)	0.1	(0.1)	0.3	(0.2)	0.0	(0.1)
TM7 genera incertae sedis	0.4	(0.4)	0.5	(0.4)	0.1	(0.2)	0.5	(0.4)	0.8	(0.6)	0.4	(0.4)
Ureaplasma	1.1	(1.2)	0.3	(0.3)	0.9	(0.9)	0.6	(0.2)	0.7	(0.6)	0.2	(0.2)
Xylanibacter	0.4	(0.2)	0.3	(0.3)	0.2	(0.1)	0.3	(0.2)	0.3	(0.1)	0.9	(0.7)

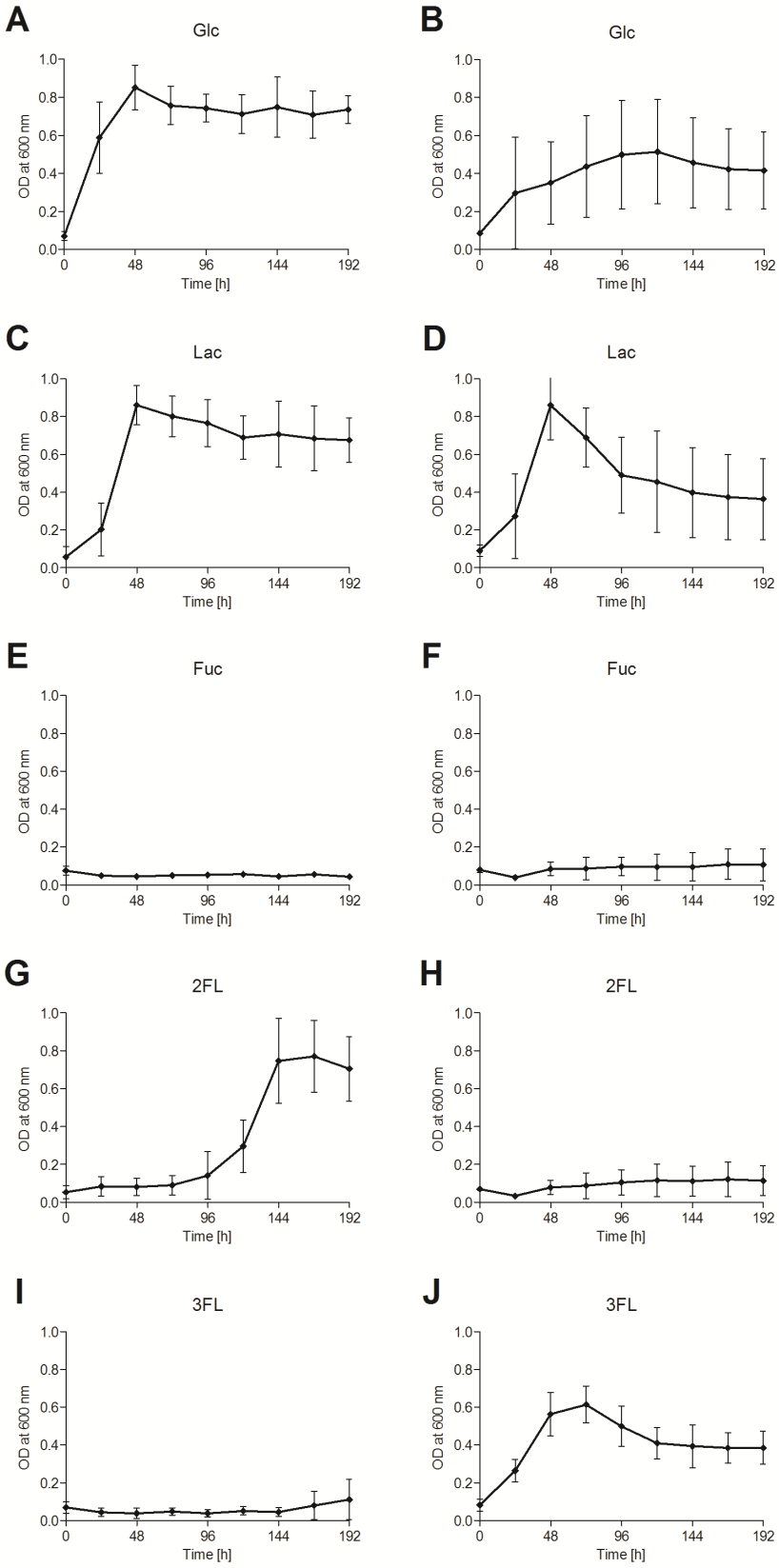

Figure 4: Selective growth of *Barnesiella intestinihominis* (left panel) and *Barnesiella viscericola* (right panel) with different sugar substrates. The two *Barnesiella* strains were incubated in YCFA medium supplemented with (A, B) glucose, (C, D) lactose, (E, F) fucose, (G, H) 2FL or (I, J) 3FL. Proliferation was measured spectrophotometrically at 600 nm. Values are mean and standard deviation of two experiments each carried out in triplicate.

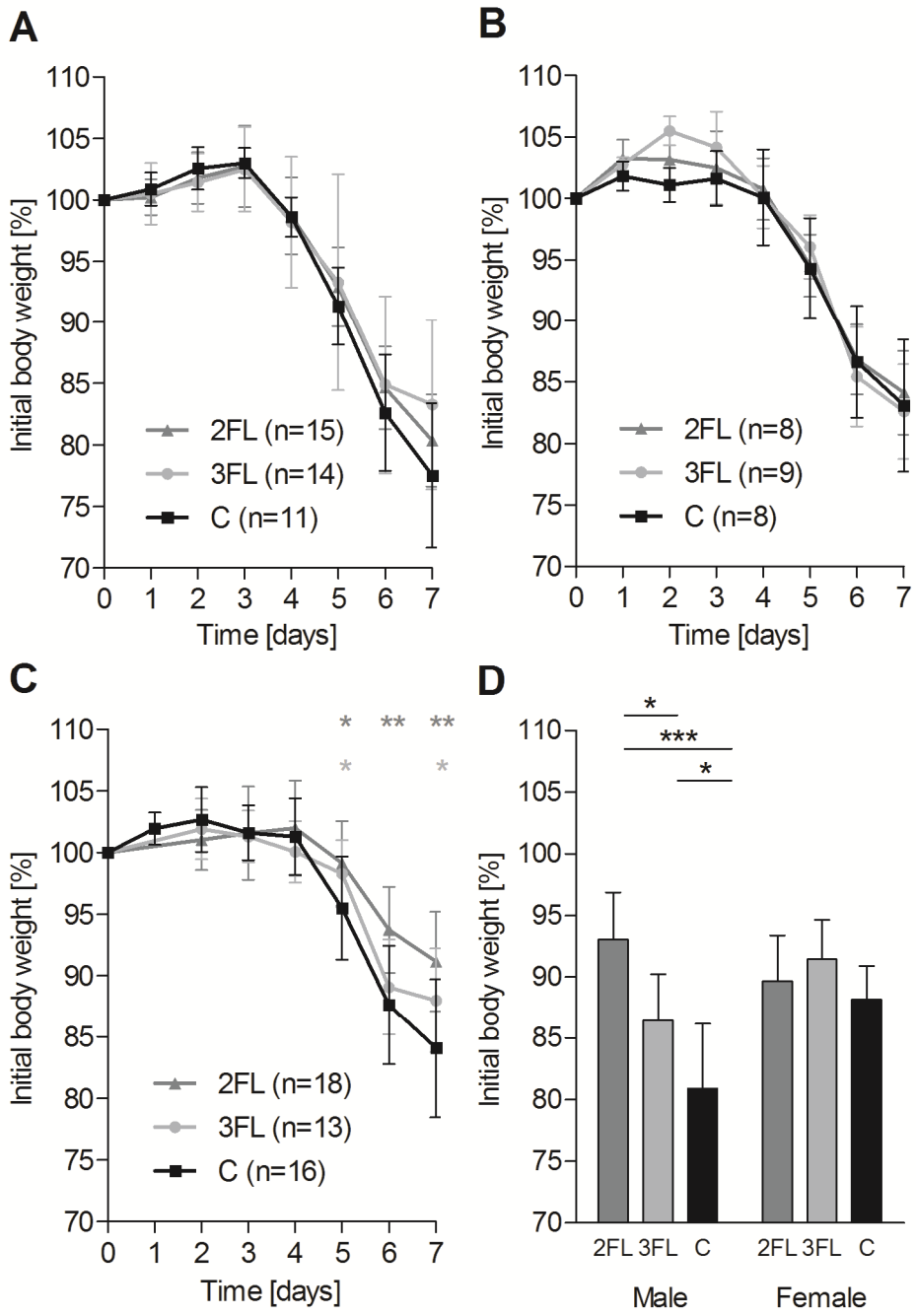

Figure 5: Susceptibility to acute colitis of fucosyllactose supplemented mice. Acute colitis in fucosyllactose-supplemented and control mice was induced by DSS treatment at the age of six weeks. Treatment with DSS in drinking water was started on day 0. On day 5, treatment was ended by changing to normal drinking water. Loss of body weight was monitored during DSS treatment and 2 subsequent days in mice supplemented (A) for 3 weeks until weaning, (B) for 3 weeks after weaning and (C) for 6 weeks (long-term supplementation). (A) and (C) present pooled data from two independent experiments. C, control. *, $P < 0.05$ vs. control by t-test; **, $P < 0.001$ vs. control by t-test. (D) Body weight loss of male and female mice is compared on day 7 after start of DSS treatment of long-term supplemented mice. Graph shows pooled data from two independent experiments. For males, n = 8, 9 and 9, for females, n = 10, 4 and 7 (2FL, 3FL and control (C) group, respectively). *, $P < 0.05$ by one-way ANOVA; ***, $P < 0.0001$ by one-way ANOVA.

7.4 Discussion

Our study demonstrated that the exposure of young mice to the fucosyllactose isoforms 2FL and 3FL, which are absent in mouse milk, affects the composition of intestinal microbiota. Specifically, the amount of *Barnesiella* increased after six weeks of fucosyllactose supplementation. *Barnesiella*, a genus of the family of *Porphyromonadaceae*, order *Bacteroidales*, was one of the most abundant microbiota detected in mouse intestine. Already by three weeks of age, it accounted for up to 25% of total intestinal microbiota. At the age of six weeks, this proportion increased to around 40% for control mice, whereas the abundance of *Barnesiella* rose close to 60% in 2FL-supplemented mice. Members of the order *Bacteroidales* contribute to carbohydrate degradation and prevent by competition the colonization of the intestine by pathogenic bacteria (446). Specifically, *Barnesiella* are able to eliminate and protect against the intestinal dominance of antibiotic-resistant pathogenic bacteria which can be observed in hospitalized patients (447). The abundance of *Barnesiella* correlates with the amount of several immunoregulatory cells. The higher the amount of *Barnesiella* in the colon, the more marginal zone B cells and invariant natural killer T cells were enumerated in spleen and liver (448). Furthermore, a direct association between a change in microbiota in favor of *Barnesiella* and the resistance to arthritis has been demonstrated in mice (449). Arthritis-resistant mice were characterized by microbiota enriched in *Barnesiella*, *Bifidobacterium* and *Parabacteroides* spp. with *Barnesiella* being the most abundant genus. In the development of colitis in IL-$10^{-/-}$ mice, higher levels of a *Barnesiella* phylotype correlated with lower activity levels of the disease (450). In the last years, many new sequences were isolated from human and animal intestine and assigned to the genus *Barnesiella* (451, 452). It still remains to be determined how the abundance of *Barnesiella* is linked to inflammatory diseases of the gastrointestinal tract.

As demonstrated in the present study, *Barnesiella intestinihominis* and *Barnesiella viscericola* can utilize fucosyllactose as energy source. This phenotype was contingent on the expression of linkage-specific fucosidases based on the respective specific utilization of 2FL and 3FL by both *Barnesiella* species. *Barnesiella intestinihominis* had been previously tested negative (444) and *Barnesiella viscericola* positive for α-fucosidase (445), although the linkage specificity of this activity was not addressed. The expression of glycosidases able to cleave carbohydrates capping lactose brings a selective advantage for bacteria competing for energy in the densely populated gastrointestinal environment. The gut of the breast-fed newborn is enriched for *Bacteroides* and *Bifidobacterium* species that possess α-fucosidase and α-sialidase activity (220, 221, 432, 453). Since α-fucosidase and α-sialidase conduct the first step of metabolizing milk oligosaccharides by removing terminal monosaccharides, the expression of these two enzymes leads to the successful intestinal establishment of species such as *Bifidobacterium infantis*, *Bifidobacterium bifidum*, *Bacteroides*

fragilis, *Bacteroides thetaiotaomicron* in the breast-fed child (214, 215, 220). Bacteria that do not exhibit α-fucosidase or α-sialidase activity, e.g. *Bifidobacterium adolescentis* and *Bifidobacterium animalis*, are not able to grow solely on milk oligosaccharides, and therefore, do not belong to the typical infant-associated microbiota (454). Little is known about the carbohydrate metabolizing activity of *Barnesiella*, but the feeding of obese mice with oligofructose has been shown to increase the abundance of *Barnesiella* by 26% (455).

The differential susceptibility of mice towards DSS-induced colitis indicated that the shift in microbial composition elicited by 2FL and 3FL supplementation had a significant biological impact on the health of the treated animal. The decreased susceptibility to DSS correlated with the levels of *Barnesiella* in 2FL and 3FL supplemented mice, suggesting that *Barnesiella* could render the intestinal milieu less prone to inflammation. The protective effect of 2FL supplementation was strongest in male mice, whereas long-term supplemented female mice did not differ significantly in their reaction to DSS from female controls. Future experiments will examine whether the distribution of specific bacterial groups such as *Barnesiella* present gender differences after fucosyllactose supplementation. Along this line, differences in the intestinal microbiome of male vs. female mice were reported in the context of an arthritis susceptibility study, in which male mice had higher amounts of *Barnesiella viscericola* than female mice (449).

Our study demonstrated that supplementation with specific human milk oligosaccharides influenced the development of intestinal microbiota in mice as shown by the changes in abundance of *Barnesiella*. Although *Barnesiella* belongs to the major bacterial genera in mice, their representation in human microbiota is minor (446, 456) and changes in *Barnesiella* have not been associated with resistance to gastrointestinal disorders in humans. Nevertheless, supplementation of 2FL might be of interest for children whose mothers lack 2FL in breast milk. Approximately 20% of Caucasians have a genetic inability to express α1,2-fucosyltransferase activity. In women, this results in the lack of α1,2-linked fucose on milk oligosaccharides, thereby to 2FL deficiency in milk (45, 288). Since α1,2-fucosylated oligosaccharides inhibit the attachment of several enteric pathogens, the absence of these glycans is associated with different infectious diseases (183, 235, 285, 457). Current research focuses on the synthesis of fucosyllactose by *Escherichia coli* (265) or with the help of bacterial enzymes (458, 459) for the production of food additives. To prevent disadvantages of children of so called non-secretors, especially in the context of necrotizing enterocolitis, the supplementation of 2FL might become a possible treatment.

7.5 Materials and Methods

Fucosyllactose supplementation in mice

Newborn C57BL/6 mice were supplemented orally with 500 mM 2FL and 3FL (Glycom) daily from day 1 to day 20 after birth starting with 5 µl, increasing by 2.5 µl every 2-3 days reaching a daily amount of 25 µl on day 20. Sterile water was given as negative control. For supplementation after weaning, 2FL and 3FL were administered in drinking water (3.3 mM) from day 21 to day 45. Fresh feces were collected on day 21 and day 45 and immediately stored at -80 °C until further analysis.

DSS-induced colitis

From day 45 postpartum, 2.75% (wt/vol) of DSS (MP Biomedicals, Cat. no. 0216011090) was added to drinking water for 5 days followed by 2 days of normal drinking water until mice were sacrificed. Body weight was monitored daily. Animals reaching less than 85% of the initial body weight were euthanized immediately. Experiments were performed in compliance with the Swiss Animal Protection Ordinance and approved by the local veterinary authority (Kantonales Veterinäramt Zürich, Switzerland).

Bacterial DNA extraction and amplification

DNA was isolated from feces samples using the QIAamp DNA Stool Mini kit (QIAGEN, Cat. no. 51504) according to the manufacturers' instructions. DNA concentrations of extracts were measured by NanoDrop (Witec AG). Aliquots of 100 ng of extracted DNA were subjected to PCR using the 16S rDNA universal primers HDA1-GC 5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAGT-3' and HDA2 5'-TTACCGCGGCTGCTGGCA-3'at 56 °C for strand annealing. Initial denaturation at 94 °C for 4 min was followed by 30 cycles of 30 s at 94 °C, 30 s at 56 °C and 1 min at 72 °C. The quality of PCR products were verified by agarose gel electrophoresis.

DGGE and species identification

Amplified 16S rDNA fragments were separated by DGGE using an INGENYphorU (Ingeny International BV) system equipped with 6% polyacrylamide gels in range of 30 to 55% denaturant, where 100% denaturant is equivalent to 7 M urea and 40% formamide. Electrophoresis was performed at 130 V for 4.5 h at 60 °C. Polyacrylamide gels were stained with GelRed™ nucleic acid stain (Biotium, Cat. no. 41003) for 45 min, destained in ultrapure water and viewed under UV. Bands of interest were

excised from gels and lysed in ultrapure water. Extracted DNA was re-amplified using the same primers and PCR conditions. To purify the bacterial DNA, PCR products were re-loaded on a denaturant gradient gel followed by excision and lysis of selected bands. DNA samples recovered from lysed bands of the second DGGE were re-amplified by PCR prior to purification by QIAquick PCR purification kit (QIAGEN, Cat. no. 28104) and sequenced (Microsynth AG, Switzerland). Species identification was performed using the Ribosomal Microbiome Database Project classifier tool (460).

Pyrosequencing and data processing

The V5-V6 region of 16S rDNA was amplified from feces DNA samples using specifically designed primers and amplicons were pyrosequenced using a Roche 454 GS-FLX system by DNAVision in Gosselies, Belgium. Bacterial diversity was determined at phylum, family and genus levels. Sequences were aligned to 16S ribosomal RNA of *Barnesiella intestinihominis* and *Barnesiella viscericola* with BLASTN 2.2.27+ (461). The GenBank accession numbers for the 16S rRNA sequences of *Barnesiella intestinihominis* and *Barnesiella viscericola* are AB267809.1 and AB370251.1, respectively.

Bacterial culture

Strains of *Barnesiella viscericola* and *Barnesiella intestinihominis* were obtained from Leibniz-Institut, Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) GmbH (Braunschweig, Germany). The cellular morphology was inspected visually by microscopy (DM1000 10X/20 microscope, Leica). Substrate utilization was determined by adding sterilized, O_2-free sugar solutions of glucose, lactose, fucose, 2FL or 3FL to a final concentration of 2.44 g/l to yeast extract, casitone, fatty acid (YCFA) medium (462). YCFA medium was dispensed in 8 ml aliquots in Hungate tubes sealed with butyl rubber septa. Short chain fatty acids were added to the medium in the final concentrations of 32.4 mM acetate, 4.7 mM butyrate, 8.7 mM propionate, 1 mM n-valeric acid, and 1.2 mM isobutyric acid. YCFA medium was prepared and maintained anaerobically using CO_2. Inoculated tubes were incubated at 37° C. Growth was measured spectrophotometrically at 600 nm.

Statistical analysis

Values are presented as mean% with standard deviation or as indicated. Comparisons of means were done by t-test or one-way ANOVA using SPSS 20. P-values lower than 0.05 were considered significant.

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Competing interests

The authors, Adrienne Weiss, Christophe Chassard and Thierry Hennet, declare no conflicts of interest.

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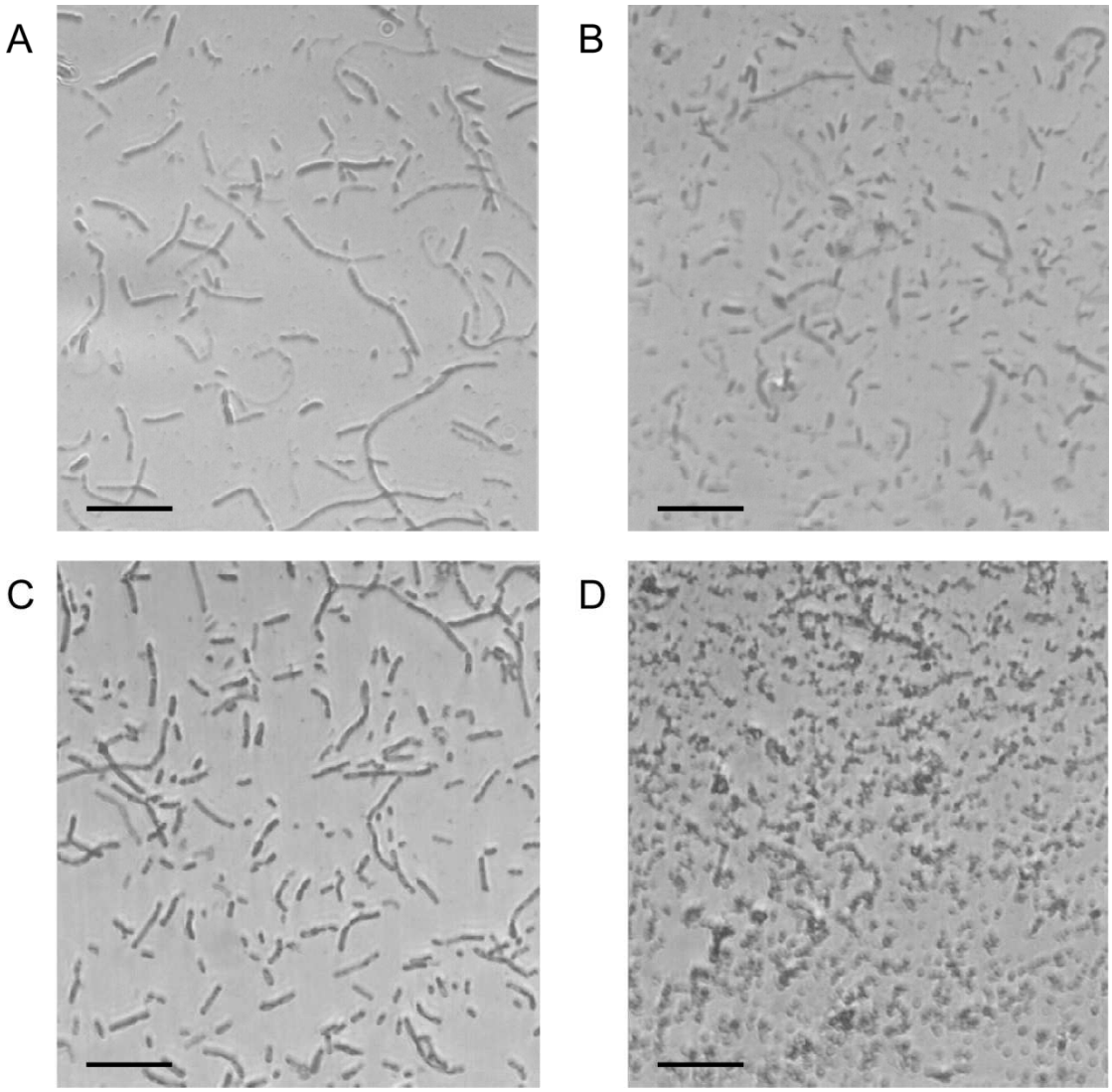
7.7 Supplemental data



Supplementary figure 1: Cell morphology of *Barnesiella* strains. Purity of the bacterial strains was tested by Gram staining for *Barnesiella intestinihominis* (left panel) and for *Barnesiella viscericola* (right panel) (A, B) before substrate utilization tests and (C, D) after four times of subsequent inoculation in YCFA medium. Bar = 20 µm.

General Discussion

8 Discussion

In this work, human milk was examined on different levels. First, content and lactational changes of fatty acids, selected lipid mediators and milk oligosaccharides were determined. Second, the impact of specific milk oligosaccharides, two isoforms of fucosyllactose, on the intestinal microbiota was investigated. The results indicate that the amount of certain milk oligosaccharides and specific fatty acids of human milk change over lactation, especially in the first weeks after birth. The developmentally important fatty acid DHA shows higher relative amounts at the beginning of lactation, like most of the measured milk oligosaccharides do. A decrease of oligosaccharides over longer periods of lactation such as one year (429) and four month (44, 463) has been reported before. These higher concentrations right after birth demonstrates the importance of DHA and milk oligosaccharides especially in early development. While DHA is needed for brain and retina development, milk oligosaccharides might support the initially immature immune system of the newborn infant.

The hypothesis of increased immune support by human milk early after birth is supported by the fact that the concentrations of many other protective milk components are also elevated at the beginning of lactation. Levels of immunoglobulins, lactoferrin and lysozyme are higher in colostrum, the milk of the first one to three days, than in mature milk (464, 465). Higher concentrations of secretory IgA and other immune factors positively correlate with higher DHA levels in breast milk (52). Thus, higher levels of both are seen in colostrum as well as in preterm milk (466, 467). Hence, the mammary gland seems to adjust its milk production in case the newborn has higher requirements. Higher levels of DHA and other protective factors in preterm milk fulfill the increased demands for these components of the preterm child (102, 105, 468). Preterm human milk not only provides significantly more DHA, but also significantly higher amounts of milk oligosaccharides than term milk (439, 469). This suggests a particular relevance of milk oligosaccharides for the preterm infant harboring an immature, not yet fully functioning immune system and an intestinal microbiota different to the one of a term infant (470).

A woman's individual milk oligosaccharide composition presumably affects the colonization of her infants' gut by bacteria. As our results indicate, especially fucosylated oligosaccharides like fucosyllactose might play a role in the growth of bifidobacteria. 2FL is the most abundant oligosaccharide in human milk and was shown to be preferentially consumed by cultured fecal microbiota that were supplemented with purified human milk oligosaccharides (293). Among

selected milk oligosaccharides, 2FL best promoted the growth of two representative bifidobacteria species of the infant gut, *Bifidobacterium longum* spp. *infantis* and *Bifidobacterium longum*. In contrast, *Escherichia coli* and *Clostridium perfringens* hardly utilized this oligosaccharide for energy consumption. 2FL belongs in general to the group of milk oligosaccharides that are rapidly fermented by microbiota of infant fecal inocula (431). One important role of 2FL has been discovered in the inhibition of pathogen binding. Here, the beneficial effect of high 2FL levels in breast milk on *Campylobacter jejuni* infection of the infant is well documented (303). However, the isolated effect of fucosyllactose on the commensal intestinal microbiota composition had not been investigated so far in vivo. This led to the selection of fucosyllactose for our intervention study in mice. The supplementation with the two forms of fucosyllactose resulted in a change of the intestinal microbiota. This change was not seen in bifidobacteria, since no bifidobacteria were detected in the murine microbiota, but in the family of *Barnesiella*. *Barnesiella* belong to the most abundant species in the murine gut and their proportion increased with fucosyllactose supplementation.

When testing the substrate utilization of selected *Barnesiella* strains, the two forms of fucosyllactose, 2- and 3FL, showed very distinct capabilities in promoting growth of the two *Barnesiella* species. This different influence of 2- and 3FL on growth was also previously observed for two *Bifibacterium longum* strains in humans (293). These observations support the finding that every human milk oligosaccharide has a different potential to promote bacterial growth (218). Therefore, the individual differences in milk oligosaccharide profiles probably result in different infant microbiota. The changes in the microbiota composition observed in our mice study might influence the immune response and mediate the observed inflammation-reducing effect of 2FL in DSS-induced colitis.

Functional studies of isolated milk oligosaccharide structures are always carried out with the ulterior motive to disclose benefits of these structures and make them available to patients and newborns that would profit from them. A possible application is the treatment of gastrointestinal diseases including chronic inflammation and bacterial infections causing diarrhea. In preterm newborns, the administration of milk oligosaccharides might lower the risk of developing NEC (436). Another aim is to provide the health advantages associated with breast-feeding to newborns that are fed infant formula. Milk oligosaccharides lower the amount of stimuli for the neonatal immune system. This anti-inflammatory effect of milk oligosaccharides can be looked at from two angles. On the one hand, it is a positive effect, since the breast-fed newborn is protected from a hyperactive immune system and hence, an exaggerated immune response. On the other hand, the immune system might be compromised as its actions are reduced. But the observation of less inflammatory diseases in human milk-fed children than in formula-fed ones supports the assumption that the overall anti-

inflammatory effect of human milk oligosaccharides offers more advantages than disadvantages (275).

One step to imitate the immune modulatory effect of human milk is the addition of galacto- and fructooligosaccharides to infant formula. These artificial alternatives may render the microbiota more bifidogenic. But human milk oligosaccharides have many other effects beyond promoting bifidobacteria. Lots of these effects are still unknown and are likely to be structure-specific such as in the inhibition of *Campylobacter jejuni* infection by α1,2-fucosylated oligosaccharides (235). Many functions rely on fucosylation or sialylation, like the reduced selectin-mediated leukocyte-adhesion or the protection from NEC (48). The oligosaccharides added to infant formula nowadays, whose structures of Gal and fructose oligomers do not occur naturally in human milk, cannot fulfill these functions, since they contain neither fucose nor Sia.

A new element of the anti-inflammatory activities of human milk was discovered in the course of this work: the presence of anti-inflammatory and pro-resolving lipid mediators and their related hydroxy fatty acids. They were detected in human milk in amounts exceeding reported plasma values by up to 100-fold. These high concentrations bear the potential to have a direct influence on the child's inflammatory status. The lipid mediators from human milk might constantly lower the inflammation during the time of breast-feeding. Due to studies showing positive treatment effects with specific lipid mediators in diverse pathophysiological conditions, they are discussed as potential therapeutics (471-473). The tissue concentration of lipid mediators greatly depends on the levels of their precursors. Therefore, an increase of precursor concentrations already exerts positive effects. This was shown for resolvins and protectins in *fat*-1-transgenic mice (418). These *fat*-1-transgenic mice have higher stores of DHA and EPA and, resulting from this, higher levels of resolvins and protectins. Interestingly, these mice show reduced gastrointestinal inflammation.

In non-transgenic animals and also in humans, the internal DHA and EPA stores depend on the dietary fatty acid pattern and represents an example how nutrition can influence the inflammatory activity. Therefore, the decision between human milk containing DHA and EPA and infant formula without these two fatty acids might have consequences for the immune response of the child. The dietary fatty acid intake determines the fatty acid composition of cell membrane phospholipids (474-476). These membrane phospholipids deliver the fatty acid precursors for the biosynthesis of inflammatory mediators. Therefore, the fatty acid composition of the cell membrane influences the pattern of the eicosanoid synthesis. A Western-type diet leads to a cell membrane with an AA content of approximately 20% of the fatty acids (384, 477). In contrast, the concentration of DHA is around 2.5% and of EPA less than 1%. Thus, the supply of the omega-3 fatty acids DHA and EPA for the synthesis of anti-inflammatory and pro-resolving resolvins and protectins is much lower than the

supply of the omega-6 precursor AA for the production of pro-inflammatory leukotrienes and prostaglandins. But the composition can be altered by a higher intake of omega-3 fatty acids. The supplementation with fish oil, i.e. oil with high EPA and DHA concentrations, significantly increased the DHA and EPA concentration in plasma phospholipids (475). The altered ratio of omega-6 to omega-3 fatty acids might enhance a less inflammatory phenotype by increased production of resolvins and protectins, but also by influencing inflammatory gene expression. Mice supplemented with fish oil showed lower mRNA levels of tumor necrosis factor-α and IL-1β in several studies (478-480). The different levels of the anti-inflammatory actions of omega-3 fatty acids support their importance in the daily nutritional intake. The content of omega-3 DHA and EPA is naturally high in human breast milk representing a natural anti-inflammation system for the newborn in addition to the present anti-inflammatory lipid mediators. Considering the diverse beneficial effects of DHA and EPA, their addition should be standard practice in the production of infant formula.

Conclusion and future implications

Human milk does not only provide the whole range of nutrients and bioactive components for the ideal development of the neonate, but also seems to adjust their concentrations to the needs of the neonate over time. This natural alignment cannot be fulfilled by infant formula. Even donor human milk does not provide the tailor-made composition of mother's own milk. It is widely accepted that breast milk is the gold standard for the newborn's nutrition. Still, out of diverse reasons, some mothers choose not to breastfeed or are unable to, and hence, rely on alternatives like donor human milk or infant formula. Donor human milk represents a valuable alternative to human milk and should be preferred over infant formula. The usual practice of pasteurization lowers the content of immunoglobulins, soluble CD14 and human milk B cells and T cells compared to "fresh" human milk. Also the activity of lactoferrin and lysozyme is decreased, but milk oligosaccharides are not affected in both their composition and their quantity (481, 482). Additionally, the fatty acid composition is only slightly altered by pasteurization: medium-chain unsaturated fatty acids increase and oleic acid decreases, but LCPUFA are not affected (483). Donor human milk is particularly needed for the initial provision of human milk to preterm infants, where the mother's own milk is not sufficient in quantity. However, in case the mother produces adequate amounts of milk, the own milk should always be preferred to donor human milk or formula (484) because of the content of immune components such as immunoglobulins and the higher level of milk oligosaccharides and DHA.

The solution for providing an alternative to human milk is formula. The general aim is to produce formula that more and more resembles human milk. The first step on this way has been made by adding the two essential fatty acids linoleic and α-linolenic acid as well as LCPUFAs like DHA and AA.

The regulations for infant formula composition define a maximum omega-3 LCPUFA content of 1% of the total amount of fatty acids (38). This upper limit does not impair proper DHA supply, considering that 0.33% DHA is sufficient to create a plasma DHA level in formula-fed infants similar to breast-fed infants (485). Also the demand for AA can well be covered with a defined upper limit of 1% of total fatty acids (38). Although the addition of LCPUFA to infant formula is only possible but not required by law, it should be self-evident. Many advantages are associated with the intake of LCPUFA apart from influencing the pattern of eicosanoid synthesis. Feeding an infant formula containing DHA and AA instead of a formula without those two fatty acids was shown to lower the blood pressure in children at the age of six years (486). It might also improve the infant's cognitive development as shown by better problem solving at the age of ten months in children that had received the LCPUFA-supplemented formula (487). Moreover, the addition of LCPUFAs improves the visual acuity of the infant in the first year of life (488, 489).

The oligosaccharides added to infant formula are still far from original milk oligosaccharides. In order to improve infant formula in this regard, the specific functions of individual oligosaccharides need to be further identified and selected oligosaccharides need to be synthesized on a larger scale. Furthermore, the effects of milk oligosaccharide administration need to be tested.

In this study, we showed the microbiota-changing and anti-inflammatory effect of 2FL administration in mice. It would be of interest how 2FL affects the microbiota in human newborns where bifidobacteria is one of the predominant bacterial species. As a similar supplementation study would not be possible in humans, one could take advantage of the fact that breast milk of non-secretors lacks 2FL. Newborns that receive milk of non-secretors would represent the ideal control group for investigations of effects of α1,2-fucosylated milk oligosaccharides on gut microbiota in humans. So far, the intestinal microbiota of breast-fed infants receiving non-secretor milk has not been described. Only the microbiota of adult individuals has been investigated in connection to the secretor status. In that study, the diversity of bifidobacteria in the adult gut had a higher diversity in secretors than in non-secretors (490). In contrast to non-secretors, the situation is different in non-secretor milk-fed newborns which themselves express α1,2-fucosyltransferase, therefore bear α1,2-linked fucose on mucosal glycan structures. They only lack the free α1,2-fucosylated oligosaccharides originating from breast milk. Without any intervention, a comparison of the gut microbiota from breast-fed infants of secretors and non-secretors would reveal if α1,2-linked-fucose from human milk, therefore mainly in the form of 2FL, increases the amount or diversity of bifidobacteria in breast-fed newborns.

Nowadays, some companies are able to synthesize tri- and tetrasaccharides that also occur in human milk like fucosyl- and sialyllactose. This opens the possibility of infant formula supplementation after

thorough investigation of the specific effects of these isolated structures. But previous studies in humans with isolated structures did not disclose any effects (267, 268). The fact that isolated milk oligosaccharides might not be as effective as a mix of diverse structures represents a major challenge for milk oligosaccharide supplementation of infant formula. The extraction of milk oligosaccharides from human donor milk might be a possible way to obtain this complex natural mix. The milk oligosaccharides from cow's milk are of limited suitability for supplementation. Apart from their lower abundance, they are less complex and show less variability than human milk oligosaccharides (46). The predominant type I structures in human milk have not been detected in cow's milk. Moreover, bovine milk oligosaccharides hardly contain fucose, the main decorating monosaccharide in human milk. In contrast, they are mainly sialylated, not only with NeuAc like in human milk, but also with the structurally related N-glycolylneuraminic acid, which does not occur in humans (491). The acidic fraction of bovine milk oligosaccharides showed anti-microbial and brain growth promoting effects that could be of interest (reviewed in ref. (46)). But aside from the difficult large-scale preparation, many structures typical for human milk cannot be provided by cow's milk.

Although fatty acids and maybe someday in the future also milk oligosaccharides can be added to infant formula, one factor for the perfect imitation of breast milk is still missing. Infant formula lacks protective components like lipid mediators and other immune factors that therefore remain one of the major reasons for the superiority of human milk over formula.

8.1 References

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Curriculum vitae

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02/2009 – present	University of Zurich Institute of Physiology *PhD Student* ▪ Project: Protective action of maternal milk in gastrointestinal physiology	Zurich, Switzerland
10/2008 – 01/2009	Justus Liebig University Institute of Nutritional Science *Research Assistant*	Giessen, Germany
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